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cAMP-dependent Signaling Regulates the Adipogenic Effect of *n*-6 Polyunsaturated Fatty Acids*[S]

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The effect of *n*-6 polyunsaturated fatty acids (*n*-6 PUFAs) on adipogenesis and obesity is controversial. Using *in vitro* cell culture models, we show that *n*-6 PUFAs was pro-adipogenic under conditions with base-line levels of cAMP, but anti-adipogenic when the levels of cAMP were elevated. The anti-adipogenic action of *n*-6 PUFAs was dependent on a cAMP-dependent protein kinase-mediated induction of cyclooxygenase expression and activity. We show that *n*-6 PUFAs were pro-adipogenic when combined with a high carbohydrate diet, but non-adipogenic when combined with a high protein diet in mice. The high protein diet increased the glucagon/insulin ratio, leading to elevated cAMP-dependent signaling and induction of cyclooxygenase-mediated prostaglandin synthesis. Mice fed the high protein diet had a markedly lower feed efficiency than mice fed the high carbohydrate diet. Yet, oxygen consumption and apparent heat production were similar. Mice on a high protein diet had increased hepatic expression of *PGC-1α* (peroxisome proliferator-activated receptor γ coactivator 1α) and genes involved in energy-demanding processes like urea synthesis and gluconeogenesis. We conclude that cAMP signaling is pivotal in regulating the adipogenic effect of *n*-6 PUFAs and that diet-induced differences in cAMP levels may explain the ability of *n*-6 PUFAs to either enhance or counteract adipogenesis and obesity.

The effect of dietary fat on human health is not solely a matter of quantity but depends also on the nature of the fatty acids. The current recommendation is to replace saturated fat by polyunsaturated fatty acids (PUFAs).[5] Today, more than 85% of the total dietary PUFA intake in Western diets is *n*-6 PUFAs, mainly linoleic acid, a precursor of arachidonic acid, whereas the consumption of *n*-3 PUFAs has declined (1). Since the high intake of *n*-6 has been associated with childhood obesity, concerns regarding this matter have been raised (2). However, animal studies have yielded conflicting results, with some studies demonstrating that a diet enriched in *n*-6 PUFAs decreases adipose tissue mass (3, 4), whereas others have associated intake of *n*-6 PUFAs with an increased propensity for obesity (5–7).

Adipose tissue increases in size by hypertrophy of preexisting adipocytes and recruitment and differentiation of new adipocytes from a preadipocyte population (8). The dichotomy of action of *n*-6 PUFAs in feeding experiments is mirrored by the dichotomy of the effects of arachidonic acid on fat cell differentiation *in vitro*. On one hand, arachidonic acid has been identified as one of the adipogenic components of serum and is required for induction of differentiation of 3T3-F442A cells and Ob1771 preadipose cells (9). On the other hand, arachidonic acid and its metabolites generated by cyclooxygenases (COXs) inhibit differentiation of primary preadipocytes (10), 1246 cells (11), and 3T3-L1 cells (12).

In the present study, we present data that reconcile and explain the disparate effects of *n*-6 PUFAs on adipocyte differentiation *in vitro* and *in vivo*. We demonstrate that cAMP signaling plays a pivotal role controlling the production of antiadipogenic prostaglandins. *In vivo*, the obesigenic action of *n*-6 PUFAs is determined by the balance between dietary carbohydrates and protein. A high carbohydrate/protein ratio translated into a high plasma insulin/glucagon ratio, and in this setting, dietary *n*-6 PUFAs promoted strongly adipose tissue expansion. Conversely, a high protein/carbohydrate ratio translated into a high plasma glucagon/insulin ratio and

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3.

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[5] The abbreviations used are: PUFA, polyunsaturated fatty acid; COX, cyclooxygenase; PGE_2 and $PGF_{2\alpha}$, prostaglandin E_2 and $F_{2\alpha}$, respectively; RT, reverse transcription; qPCR, quantitative PCR; GC, gas chromatography; MIX, methylisobutylxanthine; CRE, cAMP-response element; CREB, CRE-binding protein; 6-MB-cAMP, N^6-monobutyryl-cAMP.

enhanced cAMP-dependent signaling. In this setting, COX-mediated prostaglandin synthesis was enhanced, and dietary *n*-6 PUFAs decreased white adipose tissue mass. The decreased obesigenic action of *n*-6 PUFAs in mice fed a protein-rich diet did not result from increased dissipation of energy by uncoupled respiration but rather reflected increased energy expenditure in relation to gluconeogenesis and urea formation.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—3T3-L1 cells were cultured and induced to differentiate by 0.5 mM methylisobutylxanthine, 1 μM dexamethasone, 1 μg/ml insulin (MDI) as previously described (13). Fatty acids, prostaglandins, and inhibitors were dissolved in Me_2SO and added when differentiation was induced unless otherwise stated in the figure legends. Cells not treated received similar volumes of vehicle. Staining of lipid by Oil Red-O was performed as described previously (13).

Plasmids—Wild type and CRE site-mutated COX-2 promoter luciferase reporter constructs were kindly provided by Dr. H. R. Hershman (14). β-Galactosidase expression vector pCMVβ is from Clontech. Retroviral vectors pLXSN-hygro and pBabe-puro were kindly obtained from Dr. O. A. MacDougald. pLXSN-COX-1 was nondirectionally cloned as a HindIII fragment from pSVL-COX-1 into HindIII-digested pLXSN-hygro. pBabe-COX-2 was made by directional cloning of the BamHI/XbaI fragment from pcDNA3-COX-2 into BamHI/XbaI-digested pBabe-puro.

Transient Transfection—Preconfluent 3T3-L1 cells were transfected at 50–75% confluence with 0.95 μg of wild type or CRE site-mutated COX-2 promoter luciferase reporter constructs (14) and 0.05 μg of β-galactosidase expression vector for normalization (pCMVβ; Clontech) per well (6-well plates) using METAFECTENE™ (Biotex). Six hours after transfection, the medium was changed, and cells treated with vehicle (0.1% Me_2SO), 0.5 mM methylisobutylxanthine, and/or 100 μM arachidonic acid. Twenty-four hours after transfection cells were harvested, and luciferase and β-galactosidase activities were measured as described (13). Retrovirus production and transduction was performed as described earlier (13).

Prostaglandin Levels in Culture Supernatants—PGE_2 and $PGF_{2\alpha}$ were determined as described previously (15) using 1 ml of medium and following the instructions of the manufacturer of the PGE_2- and $PGF_{2\alpha}$-specific enzyme immunoassays (Cayman).

Animals—Male C57BL/6JBomTac mice ~6 weeks of age were obtained from Taconic Europe (Ejby, Denmark) and were divided into groups ($n = 6$). The mice were kept at a 12-h light/dark cycle at 22 °C. After acclimatization, the animals were fed *ad libitum* or pair-fed experimental diets. The compositions of the diets are presented in supplemental Table 1. Corn oil was chosen as the *n*-6 fatty acid source, since this oil is particularly enriched in linoleic acid, and analysis of the diet confirmed that more than 50% of the fatty acids in the diets were linoleic acid (supplemental Table 2). The diet did not contain arachidonic acid, but analysis of the fatty acid composition of red blood cells confirmed conversion of ingested *n*-6 PUFAs to arachidonic acid (supplemental Table 2). Body weight was recorded twice a week. Mice were killed by cardiac puncture under anesthesia (Dormitor (1 mg/kg body weight) and Ketalar (75 mg/kg body weight)), and serum was prepared from blood. Tissues were dissected out, freeze-clamped, and frozen at −80 °C.

Analyses in Computerized Metabolic Cages—Male C57BL/6J mice 6 weeks of age were purchased from Charles River Laboratories (Maastricht, The Netherlands). Animals received a standard chow diet (AIN93G/95). After 1 week of acclimatization in the experimental facility, the animals were fed a corn oil diet supplemented with sucrose ($n = 8$) or protein ($n = 8$) for a period of 5 weeks before the start of the metabolic cage experiments. Mice were acclimatized to the metabolic cage environment for 1 day prior to starting of the monitoring period. During the metabolic cage experiment, oxygen consumption, CO_2 production, food intake, and activity (*x*-*y*-z-axis) were measured as described elsewhere (16).

Serum Analysis—Glucose was determined enzymatically with reagents from Dialab; insulin was determined with the mouse insulin ELISA kit (EIA 3439) from DRG Diagnostics; glucagon was determined with a radioimmune assay kit (catalog number GL-32K; Linco); and prostaglandins were determined with the PGF2α immunoassay (catalog number DE1150) and PGE_2 immunoassay (catalog number DE0100) from R&D Systems.

Real Time RT-qPCR—Total RNA was purified from mouse tissue or cells using Trizol, and cDNA was synthesized and analyzed by real time qPCR using the ABI PRISM 7700 sequence detection system (Applied Biosystems) as described earlier (17). Primers for real time PCR (supplemental Table 3) were designed using Primer Express 2.0 (Applied Biosystems).

Western Blotting—Preparation of extracts from mouse tissue or whole cell dishes, electrophoresis, blotting, visualization, and stripping of membranes was performed as described (13). Primary antibodies used were goat anti-COX-1, goat anti-COX-2, rabbit anti-PGC-1α, rabbit anti-TFIIB (all from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA)), mouse anti-phospho-CREB (Upstate Biotechnology, Inc.), and mouse anti-Vimentin (DAKO). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse, anti-goat, or anti-rabbit antibodies obtained from DAKO.

Fatty Acid Composition—The fatty acids in diets and isolated red blood cells were extracted with chloroform/methanol (2:1) (v/v) and saponified and methylated using 12% BF_3 in methanol. The fatty acid composition of total lipids was analyzed on a gas chromatograph-mass spectrometer as previously described (18).

Urea and Amino Acid Levels—Liver samples were homogenized and deproteinated in 10% sulfosalicylic acid. Free amino acids and urea were analyzed on an amino acid analyzer (Biochrom 20 plus, Cambridge, UK).

Energy in Feces and Diets—The energy content was determined in a bomb calorimeter following the manufacturer's instruction (Parr Instruments, Moline, IL).

Statistics—Data represent mean ± S.E. Analysis of variance was performed by *post hoc* pairwise comparison: Student's *t* test (RT-PCR analysis), Tukey HSD test (relative eWAT weight and absolute liver weight), and Newman-Keuls test due to nonho-

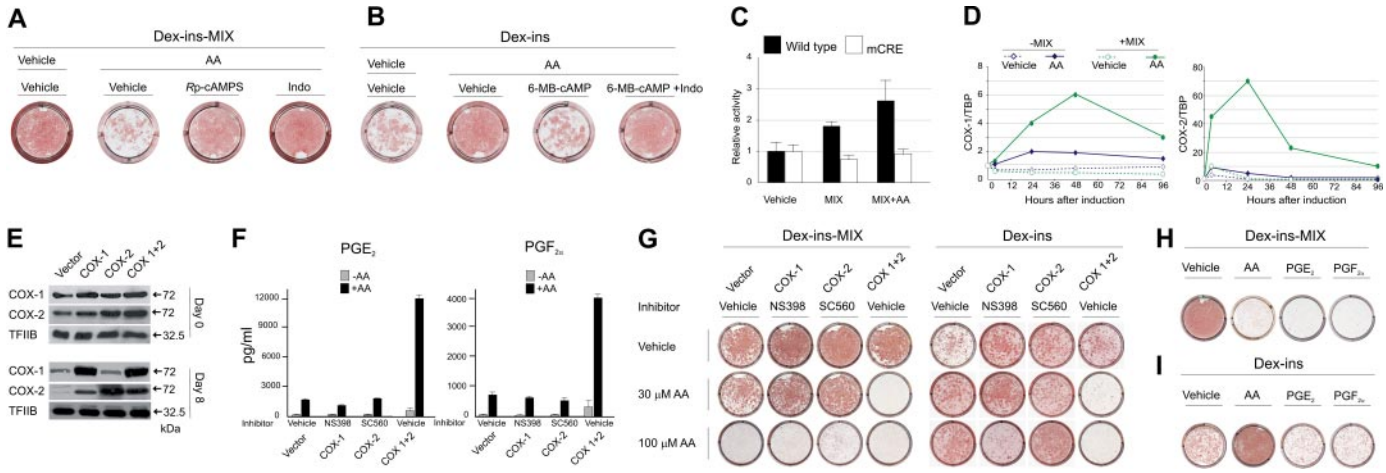

FIGURE 1. **Arachidonic acid-mediated inhibition of adipocyte differentiation requires cAMP, PKA, and cyclooxygenase activities.** 3T3-L1 preadipocytes were induced to differentiate in the absence or presence of 0.5 mM MIX. Vehicle (0.1% Me_2SO), 100 μM arachidonic acid (*AA*), 100 μM (R_p)-8-Br-cAMPS, 100 μM 6-MB-cAMP, and/or 1 μM indomethacin (*indo*) were included as indicated. Cells were stained with Oil Red-O and photographed on day 8 (*A* and *B*). Preconfluent 3T3-L1 cells were transiently transfected with wild type or a CRE site-mutated (*mCRE*) COX-2 promoter luciferase reporter and a β-galactosidase normalization vector, treated with vehicle, 0.5 mM MIX, or 0.5 mM MIX and 100 μM arachidonic acid. After 18 h, the cells were harvested for measurement of luciferase and β-galactosidase activities (*C*). 3T3-L1 cells were induced to differentiate in the absence or presence of MIX and 100 μM arachidonic acid. The cells were harvested 4, 24, 48, and 96 h after induction and analyzed for COX-1 and COX-2 mRNA expression using RT-qPCR (*D*). *E–H*, 3T3-L1 cells were retrovirally transduced with empty vector, vector encoding COX-1 or COX-2, or both. The transduced cells were induced to differentiate and analyzed for COX-1 and COX-2 expression by Western blotting at the time of induction and on day 8 (*E*). The transduced cells were induced to differentiate in presence of MIX, 30 μM arachidonic acid, and inhibitors of COX-1 or COX-2, as indicated in the figure. After 24 h, media were collected and analyzed for prostaglandin content (*F*). The transduced cells were induced to differentiate in the presence or absence of MIX. Vehicle (0.1% Me_2SO), 30 or 100 μM arachidonic acid (AA), and COX inhibitors were added as indicated. The cells were stained with Oil Red-O and photographed on day 8 (*G* and *H*). 3T3-L1 cells were induced to differentiate in the presence or absence of MIX. Vehicle (0.1% Me_2SO), 10 μM PGE_2, or 100 nM $PGF_{2\alpha}$ were included as indicated. The cells were stained with Oil Red-O and photographed on day 8 (*I*). All experiments were repeated at least three times.

mogenous variances (rest of data). Data were considered statistically significant when p was <0.05.

RESULTS

Expression of Cyclooxygenases and Production of Antiadipogenic Prostaglandins Are Regulated by cAMP in 3T3-L1 Cells—*n*-6 PUFAs, including arachidonic acid, have been reported to act both pro- and antiadipogenically *in vitro* and *in vivo* (5–7). These disparate findings may be explained by our previous finding that the effect of *n*-6 PUFAs depends on the intracellular level of cAMP (19). Accordingly, in the presence of the cAMP-elevating agent methylisobutylxanthine (MIX), arachidonic acid inhibited adipogenesis (Fig. 1*A*). Inhibition of PKA activity by (R_p)-cAMPS/(R_p)-8-Br-cAMPS or of cyclooxygenase activity by indomethacin restored adipocyte differentiation in the presence of arachidonic acid and MIX (Fig. 1*A*). By contrast, in the absence of MIX, arachidonic acid promoted adipogenesis, which, however, was inhibited by selective activation of PKA by N^6-monobutyryl-cAMP (6-MB-cAMP). Inclusion of indomethacin rescued differentiation in the presence of 6-MB-cAMP (Fig. 1*B*).

COX-2 protein expression is transiently induced when adipocyte differentiation is induced by MDI treatment (19). A cAMP-responsive element (CRE) has been identified in the COX-2 promoter (20), and hence it is likely that COX-2 expression is regulated via cAMP-dependent signaling in 3T3-L1 preadipocytes. Treatment with the cAMP-elevating agent MIX induced the expression of a luciferase reporter gene driven by the wild-type COX-2 promoter transiently transfected into 3T3-L1 preadipocytes, whereas no induction was observed when the regulatory CRE element in the COX-2 promoter was mutated. Of note, combined treatment with MIX and arachidonic acid resulted in a greater activation than treatment with MIX alone (Fig. 1*C*).

To further analyze the interplay between cAMP and arachidonic acid in regulation of COX expression, 3T3-L1 cells were induced to differentiate in the absence or presence of MIX with or without arachidonic acid, and RNA was harvested at different time points. A combined treatment with arachidonic acid and MIX led to a strong and sustained expression of both COX-1 and COX-2 during initiation of differentiation, whereas treatment with arachidonic acid or MIX alone led to a weak induction of COX-1 and COX-2 expression (Fig. 1*D*). As anticipated, omitting MIX from the induction mixture abolished the transient induction of COX-2 (and COX-1). Together, these results suggest the existence of a regulatory circuit by which cAMP/PKA-dependent induction of COX expression sensitizes the cell to an inhibitory action of arachidonic acid, which depends on COX activity, and further that the synthesized prostaglandins feed back, securing sustained expression of COX-1 and COX-2.

To corroborate the existence of such a regulatory circuit in mediating an inhibitory effect of arachidonic acid on adipocyte differentiation, COX-1 and COX-2 were retrovirally expressed, alone or in combination in 3T3-L1 preadipocytes. Retroviral expression was confirmed by Western blotting (Fig. 1*E*). Since forced expression of COX-1 induced COX-2 expression, a selective COX-2 inhibitor (NS398) was added to the COX-1-expressing cells. Similarly, cells with forced expression of COX-2 were treated with a selective COX-1 inhibitor (SC560). Twenty-four hours after the induction of differentiation, media

were collected and analyzed for the main prostaglandins produced by 3T3-L1 cells (21). Forced expression of COX-1 or COX-2 alone did not *per se* enhance prostaglandin synthesis, but when COX-1 and COX-2 were simultaneously expressed, the production of PGE_2 and $PGF_{2\alpha}$ was increased. However, exogenous arachidonic acid was required to boost the synthesis of prostaglandins (Fig. 1*F*). In accordance with this, forced expression of the COXs was not able to inhibit MDI-induced differentiation *per se* (Fig. 1*G*) but sensitized 3T3-L1 cells for arachidonic acid-mediated inhibition of differentiation, as indicated by the lack of Oil Red-O staining of cells expressing COX-1 and COX-2 treated with 30 μM arachidonic acid (Fig. 1*G*).

If the role of cAMP/PKA signaling in mediating arachidonic acid-dependent inhibition of adipocyte differentiation is solely linked to induction of COX expression, forced expression of the COXs should alleviate the requirement for elevated cAMP levels in arachidonic acid-mediated inhibition of adipocyte differentiation. In keeping with this prediction, arachidonic acid completely prevented adipocyte differentiation of 3T3-L1 cells with forced expression of COX-1 and COX-2 also in the absence of MIX (Fig. 1*G*). Finally, both $PGF_{2\alpha}$ and PGE_2 were able to inhibit differentiation in the absence or presence of MIX (Fig. 1, *H* and *I*), providing further evidence for the importance of the cAMP-PKA-COX-prostaglandin axis in regulating the effect of arachidonic acid on adipocyte differentiation.

The Effect of Corn Oil on Body Weight and Adipose Tissue Mass Is Regulated by the Balance between Carbohydrate and Protein in the Feed—As for *in vitro* studies, fundamentally opposite effects of *n*-6 fatty acids on adipose tissue development *in vivo* have been reported. Some studies have demonstrated that a diet enriched in *n*-6 PUFAs decreases adipose tissue growth (3, 4), whereas other studies have associated dietary *n*-6 PUFAs with an increased propensity to obesity (5–7). Since the adipogenic potential of *n*-6 PUFAs is dependent on the cAMP status *in vitro*, we hypothesize that the hormonal status, such as the glucagon/insulin ratio in particular, might be of importance in regulating the effect of *n*-6 PUFAs on adipose tissues also *in vivo*. Since the glucagon/insulin ratio is altered in response to intake of carbohydrates *versus* protein, we predicted that the adipogenic effect of *n*-6 PUFAs might be determined by the ratio between carbohydrates and protein in the feed.

To test this hypothesis, obesity-prone C57BL/6J mice were fed an energy-dense high fat diet enriched in *n*-6 fatty acids (corn oil), supplemented with either protein or sucrose (supplemental Tables 1 and 2) for 53 days. The C57BL/6J mice were chosen in order to limit adaptive thermogenesis that occurs in most mice strains when fed an energy-dense diet (22). Corn oil was chosen as an *n*-6 fatty acid source, since this oil is enriched in linoleic acid, the predominant PUFA in Western diets (23). Analysis of the diet confirmed that more than 50% of the fatty acids in the diets were linoleic acid (supplemental Table 2). The diet did not contain arachidonic acid, but analysis of the fatty acid composition of red blood cells confirmed conversion of the dietary *n*-6 PUFAs to arachidonic acid (supplemental Table 2). The corn oil-enriched diets were isocaloric and contained a total of 24.3 $\pm$ 0.3 and 24.9 $\pm$ 0.1 weight % fat, respectively. It should be noted that the sucrose-enriched diet contained 20 weight % protein and hence was not protein-deficient.

Mice fed the sucrose-supplemented corn oil diet *ad libitum* gained considerably more weight than mice fed the high protein-supplemented corn oil diet (Fig. 2*A*). The higher total body weight gain in mice fed corn oil in combination with sucrose was to a large extent due to an increase in white adipose tissue mass (Fig. 2*A*).

To evaluate whether the different effect of the diets could be explained by altered energy expenditure and/or voluntary activity of the animals, the mice were individually housed in metabolic cages. No difference in oxygen consumption was found between the two groups, but the respiratory exchange rate was lower in mice fed corn oil in combination with protein than with sucrose (Fig. 3), indicating that relatively more fat and possibly protein were used as substrates for oxidation. However, no increase in expression of key enzymes involved in fatty acid oxidation in muscle or liver was observed (Fig. 4). In fact, a lower heat production, indicating lower total energy expenditure, was observed in mice fed the protein-supplemented diet (Fig. 3). This was not an effect of reduced animal activity but could partly be explained by the observed lower food intake (Fig. 3).

A high protein intake is known to increase satiety and thereby to reduce energy intake (24, 25). Thus, in order to exclude the possibility that reduced adipose tissue mass in mice fed corn oil and protein *ad libitum* was simply due to reduced caloric intake, a third set of mice was pair-fed the same diets for 56 days. The mice fed corn oil in combination with sucrose gained an average of 11.3 g of body weight and became visibly obese (Fig. 2, *B* and *C*, and Table 1). The mice fed corn oil in combination with protein gained on average less than 1.8 g of body weight during the 56 days of feeding and had small amounts of white adipose tissue (Table 2 and Fig. 2, *B* and *C*). In fact, the weight gain and amount of body fat in mice fed a high corn oil diet supplemented with protein was comparable with the body weight gain and adipose tissue mass in mice fed an energy-restricted low fat chow diet (Fig. 2, *B* and *C*, and Table 1).

A Protein-enriched Corn Oil Diet Increases the Glucagon/Insulin Ratio, cAMP Signaling, and Circulating Prostaglandin Levels—Our *in vitro* results and feeding experiments suggest that the adipogenic potential of *n*-6 PUFAs can be determined by the hormonal status of the animals. In order to confirm the diet-induced hormonal differences, plasma levels of insulin and glucagon were measured. As anticipated, the levels of glucagon were higher, and the levels of insulin were lower in serum of mice fed the corn oil diet enriched in protein compared with sucrose (Fig. 2*D*). Thus, mice fed the protein-enriched corn oil diet had a glucagon/insulin ratio more than 3-fold higher than the mice fed the sucrose-enriched corn oil diet (Fig. 2*D*).

Increased glucagon/insulin ratios are known to increase intracellular levels of cAMP in both liver and adipose tissue (26–29). Reflecting the glucagon/insulin ratios, the expression of the cAMP-responsive genes cAMP-responsive element modulator and *PDE4b* (cAMP-specific phosphodiesterase 4b) was increased (Fig. 2*E*), and phosphorylation of CREB (Fig. 2*F*) was higher in inguinal white and interscapular brown adipose

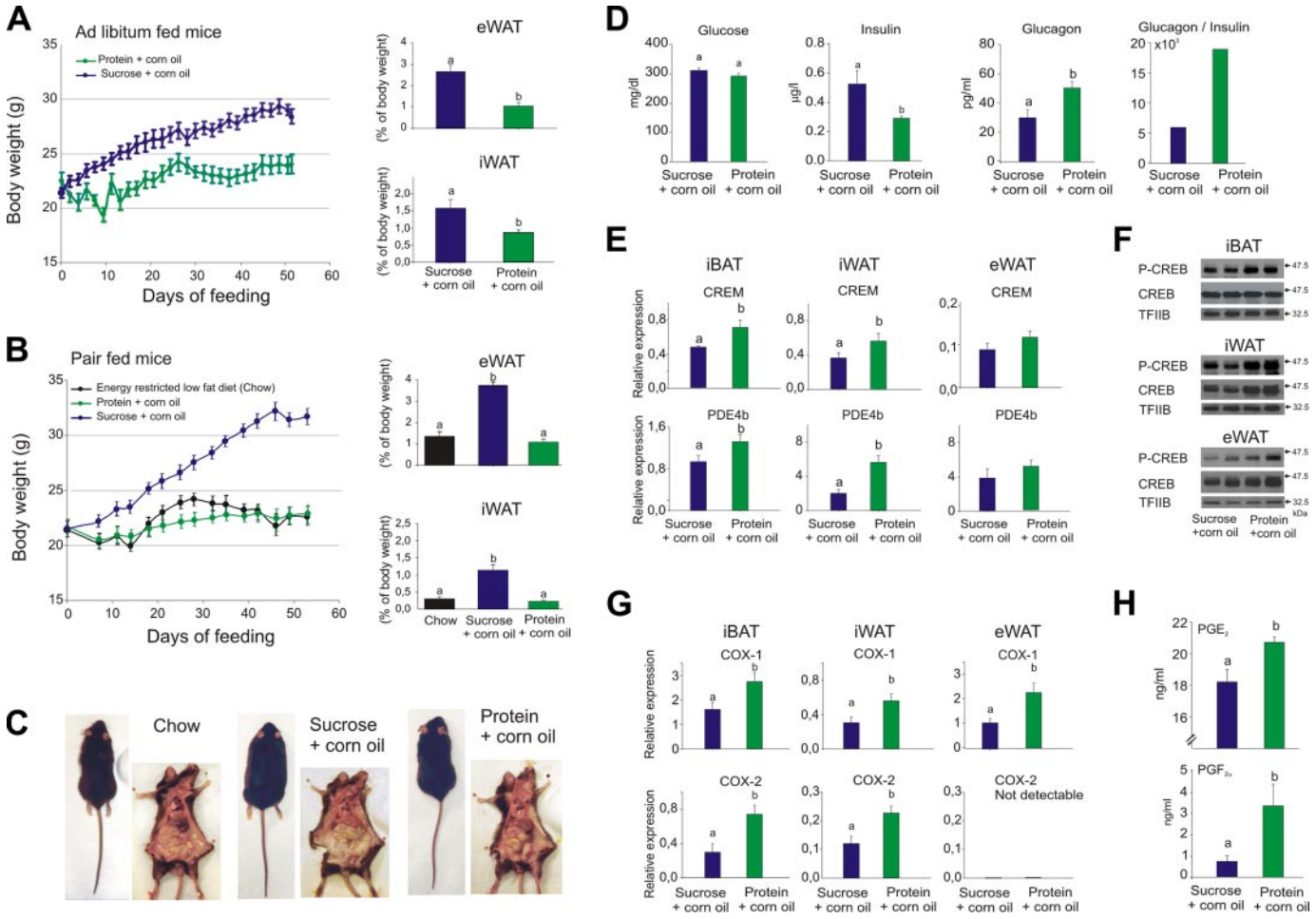

FIGURE 2. **A high corn oil diet supplemented with sucrose, but not protein, induces obesity in C57BL/6J mice.** C57BL/6J mice ($n = 6$) were fed a high corn oil diet supplemented with protein or sucrose *ad libitum*. After 53 days, the experiment was terminated, and the mice and adipose tissue were weighted (*A*). *B–H*, C57BL/6J mice ($n = 6$) were pair-fed a high corn oil-supplemented diet with protein or sucrose, whereas a third group received a normal chow diet. The weight was recorded twice per week (*B*). After 56 days, the experiment was terminated, the mice were photographed, and adipose tissues (interscapular brown adipose tissue (*iBAT*), inguinal white adipose tissue (*iWAT*), and epididymal white adipose tissue (*eWAT*)) were dissected out and weighed (*C*). *D*, glucose, insulin, and glucagon levels in serum. Expression of the cAMP-responsive element modulator (*CREM*) and cAMP-specific phosphodiesterase 4b (*PDE4b*) was measured by RT-qPCR and normalized to TATA-box-binding protein (*TBP*) in adipose tissue (*E*). Phosphorylation of CREB was determined by Western blotting. Expression of vimentin verified equal loading of protein (*F*). Expression of COX-1 and COX-2 in adipose tissues was measured by RT-qPCR. The expression in each animal was normalized to TBP (*G*). *H*, serum PGE_2 and $PGF_{2\alpha}$.

tissue in mice fed the corn oil diet enriched in protein compared with sucrose. This was accompanied by increased expression of COX-1 and COX-2 (Fig. 2*G*). Although we observed large individual differences within each group of animals, plasma levels of the antiadipogenic prostaglandins, PGE_2 and $PGF_{2\alpha}$, were elevated in mice fed the protein-enriched corn oil diet, reflecting the increased expression of COXs (Fig. 2*H*).

Enhanced cAMP signaling in interscapular brown adipose tissue is known to induce *UCP1* and allows energy to dissipate in the form of heat (30). However, in keeping with the reported resistance of C57BL/6J mice to diet-induced thermogenesis (22) and the similar heat production observed in the two groups (Fig. 3), expression of *UCP1* was comparable in interscapular brown adipose tissue of the two groups (Fig. 5). Expression of *UCP1* and *PGC-1α* was, however, higher in inguinal white adipose tissue and tended to be higher in epididymal white adipose tissue in mice fed the protein-enriched corn oil diet. The higher expression of *UCP1* in inguinal white adipose tissue was accompanied by higher expression of genes involved in fatty acid oxidation, such as *CPT1b* (carnitine palmitoyl-CoA transferase b), but the expression of these genes was still low compared with the expression in interscapular brown adipose tissue. Of note, expression of *CPT1b* and *ACO* (acyl-CoA oxidase) was also elevated in interscapular brown adipose in the mice receiving the high protein diet, whereas expression of the lipogenic genes *ACC1* (acyl-CoA carboxylase 1) and *FAS* (fatty acid synthase) was markedly decreased (Fig. 5). This may at least in part explain the low weight of interscapular brown adipose tissue in mice fed the high protein-supplemented diet (Table 2). Taken together, the analyses of gene expression and the measurements performed using metabolic cages indicate that uncoupling of mitochondria in adipose tissue at most contributed little, if anything, to the reduced adipose tissue mass in mice fed the protein-enriched corn oil diet. Furthermore, as determined by gene expression analyses, fatty acid oxidation did not seem to be up-regulated in liver and muscle. Rather, higher expression of adipocyte differentiation markers, such as peroxisome proliferator-activated receptor γ and CCAAT/en-

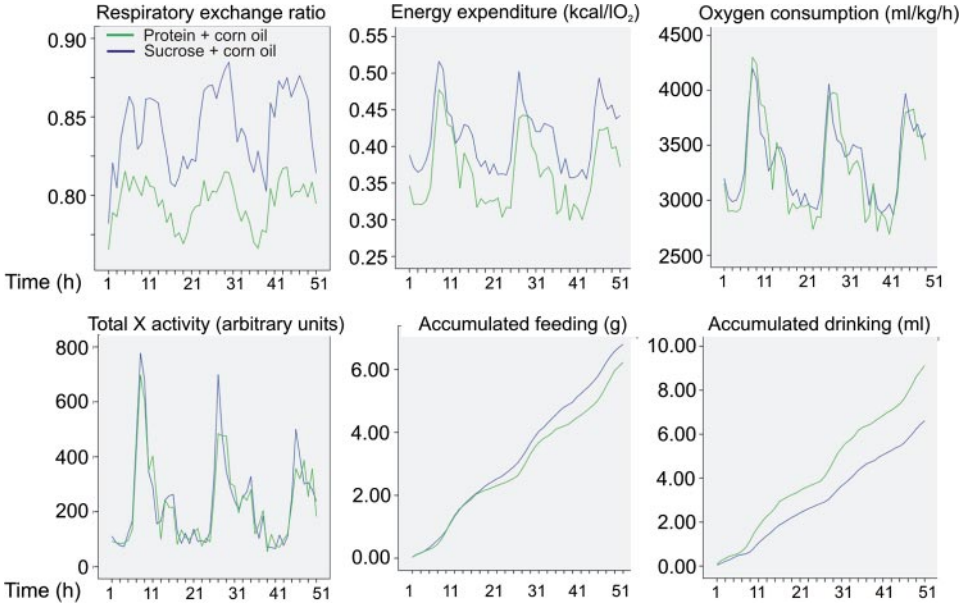

FIGURE 3. **Metabolic parameters of mice fed corn oil in combination with protein or sucrose.** Obesity-prone C57BL/6J mice ($n = 8$) were fed a high corn oil diet supplemented with protein or sucrose. After 5 weeks, respiratory exchange ratio, heat production, oxygen consumption, total X activity, accumulated feeding, and accumulated drinking were measured during a 50-h period.

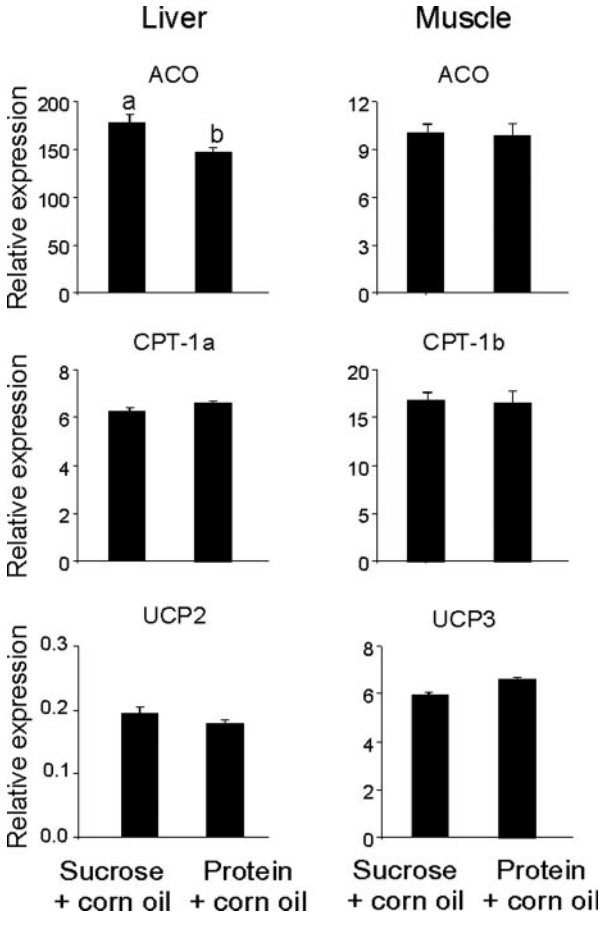

FIGURE 4. **The expression of genes involved in fatty acid oxidation is similar in liver and muscle of mice fed corn oil in combination with protein or sucrose.** Obesity-prone C57BL/6J mice ($n = 8$) were fed *ad libitum* a high corn oil diet supplemented with protein or sucrose. After 5 weeks, liver and muscle (gastrocnemius/plantaris) were collected. RNA was isolated, and cDNA was synthesized from each individual animal. Expression of *CPT1*, acyl-CoA oxidase (*ACO*), *UCP1*, *UCP2*, and *UCP3* was measured by RT-qPCR.

hancer-binding protein α, in adipose tissue of mice fed a corn oil-enriched diet supplemented with sucrose rather than protein suggested that the two diets had a differential effect on adipocyte differentiation (Fig. 5).

A Protein-enriched Corn Oil Diet Induces Expression of PGC-1α in the Liver and Increases Expression of Enzymes Involved in Urea Synthesis and Gluconeogenesis—Despite an equal intake of energy, the mice fed the protein-enriched corn oil were resistant to diet-induced obesity (Fig. 2), with no increase in oxygen consumption, thermogenesis, or voluntary activity compared with mice fed corn oil plus sucrose (Fig. 3). This immediately raised the question of where energy was dissipated in the mice on the protein-enriched diet and suggested that energy-demanding processes such as gluconeogenesis and urea production might be increased in these mice. In the liver, *PGC-1α* is induced in response to elevated levels of cAMP and plays a central role in the control of gluconeogenesis (31–33). As anticipated, a high glucagon/insulin ratio translated into induced phosphorylation of hepatic CREB and expression of canonical cAMP-responsive genes, *PGC-1α* and phosphoenolpyruvate carboxykinase (Fig. 6*A*), indicating that the capacity for gluconeogenesis was increased.

Given that certain prostaglandins can stimulate gluconeogenesis in isolated rat hepatocytes (34) and that mice fed the protein-enriched corn oil diet exhibited elevated levels of circulating $PGF_{2\alpha}$ and PGE_2, we investigated whether these prostaglandins were able to stimulate the expression of *PGC-1α* in hepatocytes using Hepa1-6 cells as a model. Indeed, PGE_2 induced *PGC-1α* as efficiently as the PKA-activating cAMP analogue 6-MB-cAMP (Fig. 6*B*). In contrast to 6-MB-cAMP, neither $PGF_{2\alpha}$ nor PGE_2 induced the expression of the cAMP-responsive enzyme cAMP-responsive element modulator (Fig. 6*B*). Thus, circulating PGE_2 may potentiate the effect of a high glucagon/insulin ratio on hepatic gluconeogenesis by further inducing *PGC-1α* expression.

Since an elevated glucagon/insulin ratio also is known to increase urea synthesis in liver (35), we determined the expression of key enzymes involved in amino acid catabolism and urea synthesis. As expected, the levels of mRNAs encoding these liver enzymes were significantly higher in the mice fed the protein-enriched corn oil diet compared with those fed the sucrose-enriched corn oil diet (Fig. 6*D*). Despite the difference in dietary protein contents in the high protein and high sucrose diets (54 and 20% by weight, respectively) (supplemental Table 1), the levels of free amino acids in the liver were similar in both feeding groups (Fig. 6*C*). In contrast to the increased expression of genes involved in the catabolism of amino acids and urea production, there was no significant differ-

TABLE 1

Feed intake, weight gain, and energy efficiency in pair-fed mice

Data represent mean ± S.E. ($n = 6$).

	Chow	High sucrose plus corn oil	High protein plus corn oil
Feed intake (g)			
Total	142.4 ± 0.7	140.9 ± 0.3	137.1 ± 2.4
Daily	2.66 ± 0.01	2.63 ± 0.06	2.56 ± 0.04
Energy intake (kcal)			
Total	634 ± 3[a]	767 ± 16[b]	842 ± 14[c]
Daily	12.0 ± 0.1[a]	14.5 ± 0.3[b]	15.9 ± 0.2[c]
Growth (g)			
Total	2.9 ± 0.9[b]	11.3 ± 1.1[c]	1.8 ± 0.7[b]
Daily	0.05 ± 0.01[b]	0.21 ± 0.02[c]	0.03 ± 0.01[b]
Weight gain/energy intake (g/Mcal)	4.5 ± 1.5[b]	14.7 ± 1.4[c]	2.2 ± 0.8[b]
Apparent energy digestibility[d] (%)	92.3 ± 0.2[b]	94.0 ± 0.2[c]	94.7 ± 0.2[c]

[a–c] Different letters indicate significant differences ($p < 0.05$).
[d] 100 × ((energy intake − fecal energy)/energy intake).

TABLE 2

Body and organ weights in pair-fed mice

Data represent mean ± S.E. ($n = 6$). BW, body weight.

	Chow	High sucrose plus corn oil	High protein plus corn oil
Final BW (g)	24.2 ± 0.5[a]	32.8 ± 1.3[b]	23.5 ± 0.5[a]
Epididymal adipose tissues			
Total (g)	0.31 ± 0.03[a]	1.27 ± 0.15[b]	0.24 ± 0.03[a]
Percentage of body weight	1.3 ± 0.1[a]	3.8 ± 0.3[b]	1.0 ± 0.1[a]
Inguinal adipose tissue			
Total (g)	0.08 ± 0.01[a]	0.35 ± 0.06[b]	0.07 ± 0.01[a]
Percentage of body weight	0.32 ± 0.03[a]	1.03 ± 0.16[b]	0.28 ± 0.04[a]
Interscapular adipose tissue			
Total (g)	0.20 ± 0.01[a]	0.37 ± 0.05[b]	0.13 ± 0.01[a]
Percentage of body weight	0.81 ± 0.04[a]	1.13 ± 0.17[b]	0.56 ± 0.05[a]
Liver			
Total (g)	1.05 ± 0.04[a]	1.38 ± 0.08[b]	1.14 ± 0.03[a]
Percentage of body weight	4.3 ± 0.1[a]	4.2 ± 0.1[a]	4.8 ± 0.1[b]

[a,b] Different letters indicate significant differences ($p < 0.05$).

ence in liver urea levels (Fig. 6*C*), but mice fed the high protein diet had a significantly higher water intake (Fig. 2), probably reflecting a higher urine production and excretion of produced urea.

The carbon skeleton of glucogenic amino acids is a source of pyruvate for gluconeogenesis, but despite the large differences in dietary protein levels, the levels of free glucogenic amino acids in the livers were also equal (Fig. 6*C*). This may be explained by an efficient deamination of glucogenic amino acids and production of pyruvate. Considering that mice fed the high protein diet had a significantly higher intake of water (Fig. 3), these data collectively suggest that the mice fed the high protein diet had a higher urine production and excretion of the produced urea.

Gluconeogenesis is an energy-consuming process, since six ATP molecules are consumed per molecule of glucose synthesized from pyruvate. Transformation of amino acids into glucose is even more energy-demanding, since ATP is used to dispose of nitrogen as urea. This is reflected by the fact that mice fed corn oil in combination with sucrose had extremely high energy efficiencies compared with those fed the protein-enriched diet (Table 1). Furthermore, the suppression of genes involved in lipogenesis, a hallmark of PUFA action (36), was attenuated in these mice, whereas expression of enzymes involved in fatty acid β-oxidation and ketogenesis was similar (Fig. 6, *E–G*). Thus, the balance between fatty acid synthesis and oxidation is shifted toward synthesis in the sucrose-fed mice, suggesting that ingestion of sucrose abolished the normal corn oil-induced suppression of lipogenic genes, allowing lipogenesis to occur despite a high intake of dietary fat.

DISCUSSION

Contemporary Western diets contain an abundance of *n*-6 fatty acids, predominantly linoleic acid, from vegetable oils that are used in industrially prepared food (23). Concerns have been raised that the high intake of n-6 PUFAs increases the propensity for developing childhood obesity (2). The obesigenic potential of *n*-6 PUFAs has, however, been a matter of dispute, since conflicting results both *in vivo* and *in vitro* have been published. Some studies have demonstrated that diets enriched in *n*-6 PUFAs decrease adipose tissue growth (3, 4), whereas other studies have associated dietary *n*-6 PUFAs with an increased propensity to obesity (5–7). Here we show that the adipogenic potential of arachidonic acid is determined by cAMP levels in cultured preadipocytes, where conversion of arachidonic acid to the antiadipogenic prostaglandins PGE_2 and $PGF_{2\alpha}$ is mediated by a cAMP/PKA-dependent induction of COX expression. We present evidence that a cAMP-PKA-COX-prostaglandin regulatory circuit also at least in part regulates the adipogenic potential of *n*-6 PUFAs *in vivo*.

A number of observations indicated that the increased glucagon/insulin ratio observed in mice fed the protein-enriched corn oil diet enhanced cAMP-dependent signaling and PKA activation in adipose tissues. First, phosphorylation of CREB and enhanced expression of the canonical cAMP-responsive genes cAMP-responsive element modulator and *PDE4c* (cAMP-specific phosphodiesterase 4c) were increased in inguinal white and interscapular brown adipose tissue. Second,

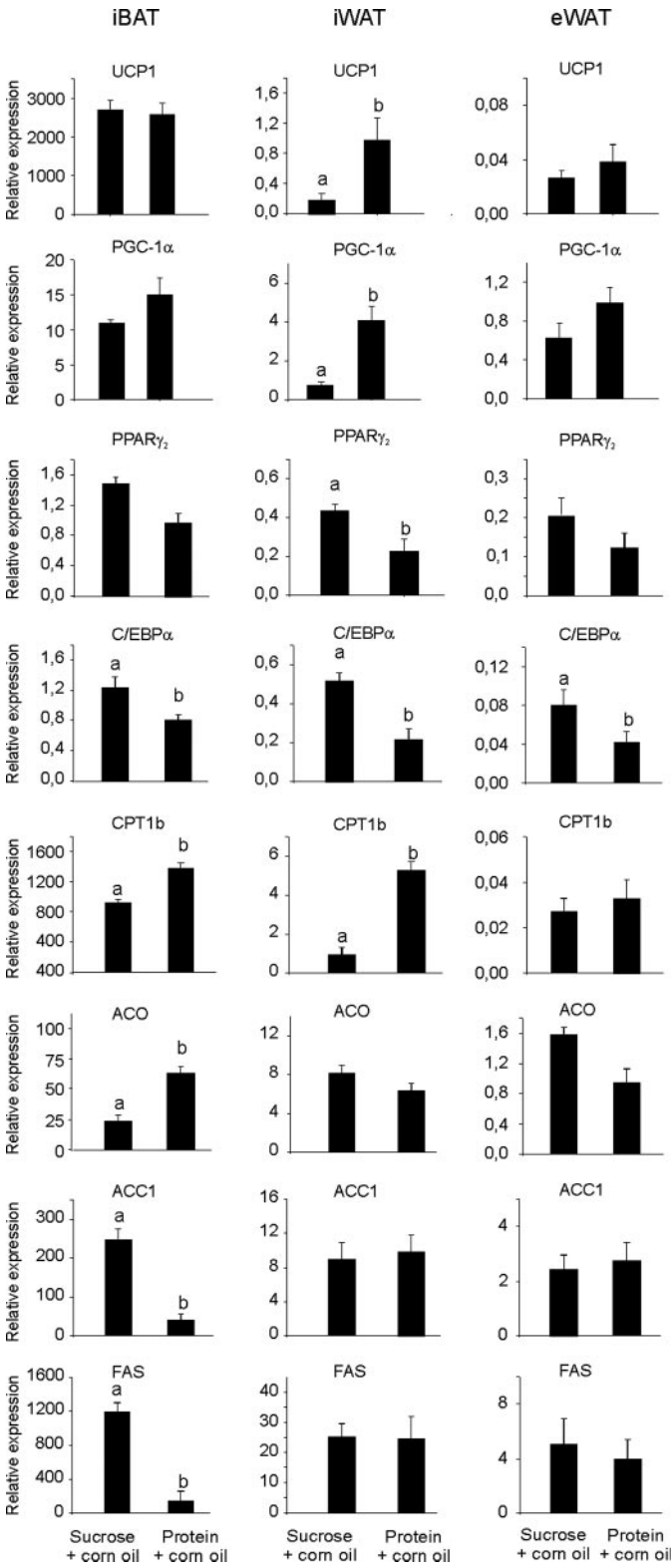

FIGURE 5. **Expression of genes involved in uncoupling, adipogenesis, β-oxidation, and lipogenesis.** C57BL/6J mice ($n = 6$) were pair-fed a high corn oil diet supplemented with protein or sucrose. After 56 days, the experiment was terminated, and adipose tissues (interscapular brown adipose tissue (*iBAT*), inguinal white adipose tissue (*iWAT*), and epididymal white adipose tissue (*eWAT*)) were collected. RNA was isolated, and cDNA was synthesized from each individual animal. Expression of *UCP1* and *PGC-1α*, *CPT1b*, *ACO*, *ACC1*, and *FAS* was measured by RT-qPCR and normalized to *TBP*.

expression of the CREB target gene COX-2 was higher in inguinal white and interscapular brown adipose tissue in mice fed the protein-enriched corn oil diet than in mice fed the sucrose-enriched diet, and the higher level of expression was paralleled by increased plasma levels of the antiadipogenic arachidonic acid-derived prostaglandins, PGE_2 and $PGF_{2\alpha}$. Of note, local overexpression of COX-2 in the skin previously has been demonstrated to result in increased prostaglandin plasma levels (37). Thus, systemic as well as local effects of PGE_2 and $PGF_{2\alpha}$ may contribute to the reduced body weight due to an inhibitory action on adipocyte differentiation, a notion also supported by lower expression of adipocyte differentiation markers, such as peroxisome proliferator-activated receptor γ and CCAAT/enhancer-binding protein α in adipose tissue. It should be noted that the relative mass of other organs remained relatively constant in the two groups of mice.

We observed a remarkable difference in feed efficiency between mice fed the protein-enriched *versus* the carbohydrate-enriched diet. In the high protein group, 467.8 kcal were needed to produce a weight gain of 1 g, whereas the high carbohydrate group only needed 67.8 kcal to produce the same weight gain, which almost exclusively represented an increase in adipose tissues. Increased cAMP signaling is known to induce adaptive thermogenesis by inducing expression of *PGC-1α* and *UCP1* in brown adipose tissue (30), but the fact that heat production and oxygen consumption as well as expression of *UCP1* in intracapular brown adipose tissue were similar in the two groups of mice indicated that decreased feed efficiency of the protein group was not due to increased uncoupled respiration. Furthermore, no increase in genes involved in fatty acid oxidation in muscle and liver was observed in the mice fed the protein-enriched diet, and the total physical activity of the carbohydrate and the protein group did not differ. Expression of *UCP1* and genes involved in β-oxidation was, however, induced in the inguinal fat pad, but the relatively low expression of these genes compared with interscapular brown adipose tissue suggested that such a contribution to whole body metabolism was limited.

Hepatic PGC-1α is a central target of the insulin/glucagon axis regulating the activation of the entire gluconeogenesis program in liver (31–33). Gluconeogenesis requires ATP, and activation of gluconeogenesis reduces feed efficiency as protein is converted to glucose at a cost of 4–5 kcal/g protein (38). Moreover, increased catabolism of amino acids requires ATP to dispose of nitrogen as urea at an energy cost of 1.33 kcal/g urea. Collectively, increased phosphorylation of CREB and induction of hepatic cAMP-responsive genes, including *PGC-1α* and phosphoenolpyruvate carboxykinase, suggest that gluconeogenesis is induced in response to a protein-induced increase in the glucagon/insulin ratio. An elevated glucagon/insulin ratio is also known to increase urea synthesis in liver (35). Consistent with this notion, we observed an induction of mRNAs encoding enzymes involved in catabolism of gluconeogenetic amino acids and urea production as well as increased water intake in the protein-fed mice.

A hallmark of PUFA action is the ability to increase catabolism by enhancing ketogenesis and peroxisomal and mitochondrial fatty acid oxidation and to suppress expression of genes

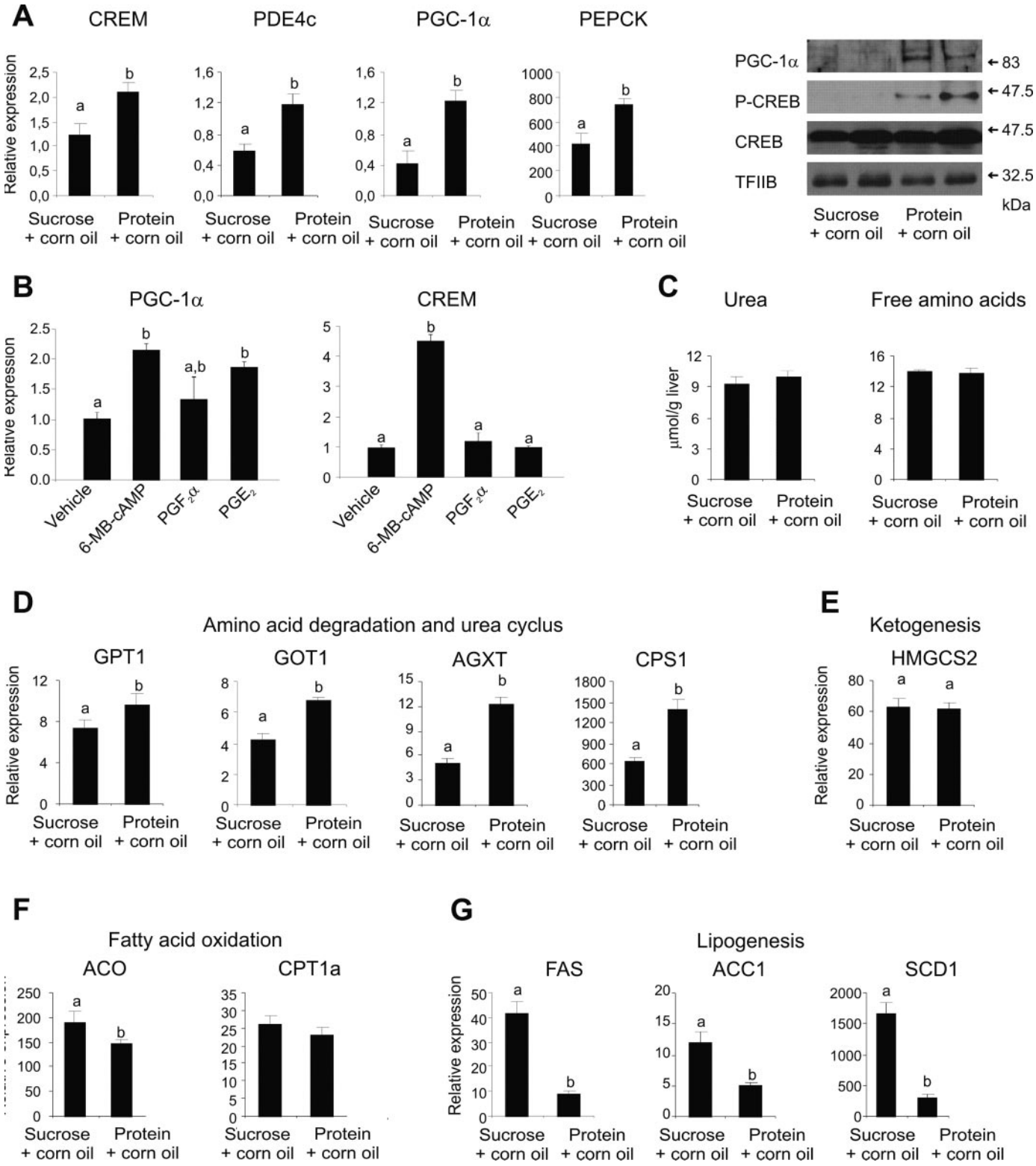

FIGURE 6. **cAMP signaling and expression of *PGC-1α* and gluconeogenic genes are elevated in the livers of mice fed corn oil supplemented with protein.** C57BL/6J mice ($n = 6$) were pair-fed a high corn oil diet supplemented with protein or sucrose for 56 days. Expression of cAMP-responsive element modulator (*CREM*), *PDE4c*, peroxisome proliferator-activated receptor γ coactivator 1α (*PGC-1α*), and phosphoenolpyruvate carboxykinase (*PEPCK*) was measured by RT-qPCR and normalized to *TBP*. Protein was extracted from liver samples and analyzed for phospho-CREB and PGC-1α using Western blotting. Expression of TFIIB verified equal loading of protein (*A*). Hepa1-6 cells were treated with vehicle, 6-MB-cAMP (100 μM), $PGF_{2\alpha}$ (10 nM), or PGE_2 (1 μM) for 6 h. RNA was isolated, and cDNA was synthesized from triplicates. Expression of *PGC-1α* and CREM was measured by RT-qPCR and normalized to *TBP*. The results represent the mean ± S.E. ($n = 3$) (*B*). The levels of free amino acids and urea in liver were determined as described under "Experimental Procedures" (*C*). Expression in liver of glutamic pyruvate transaminase (*GPT1*), glutamate oxaloacetate transaminase (*GOT1*), alanine glyoxylate transaminase (*AGXT*), and carbamoyl-phosphate synthase (*CPS1*) (*D*); mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*HMGCS2*) (*E*); *ACO* and *CPT1a* (*F*); and *FAS*, *ACC1*, and *SCD1* (stearoyl-CoA desaturase 1) (*G*) was measured by RT-qPCR and normalized to *TBP*.

involved in lipogenesis in rodents (36). It is worth noting that the hepatic expression of rate-limiting enzymes involved in fatty acid catabolism was similar in mice fed corn oil supplemented with protein and sucrose. In contrast, expression of genes involved in lipogenesis was significantly lower in liver of mice fed corn oil and protein compared with corn oil and sucrose. Thus, despite high dietary intake of fatty acids, expression of genes involved in *de novo* synthesis of fatty acid continued when dietary corn oil was combined with sucrose.

In conclusion, we have shown that the adipogenic potential of *n*-6 PUFAs is modulated by cAMP signaling both *in vivo* and *in vitro*. Differences in culture conditions and feeding regimes affecting the glucagon/insulin ratio provide an explanation for the contradictory results published in the literature. Today's diets are abundant in *n*-6 fatty acids from vegetable oils (corn, sunflower, safflower, and soybeans) that are used in industrially prepared food. In addition, industrially produced animal feed is also rich in grains containing *n*-6 PUFAs, leading to meat enriched in *n*-6 PUFAs at the expense of *n*-3 fatty acids (39). *n*-6 PUFAs, predominantly linoleic acid, are now the predominant source of PUFAs in Western diets (23). PUFAs have been considered less harmful to human health than saturated fat, and substitution of saturated fat with PUFAs in general has been recommended by dieticians. If the background diet determines the adipogenic potential of *n*-6 PUFAs also in humans, this is of great concern, since the intake of refined sugars from sources such as soft drinks has increased dramatically during recent decades (40).

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cAMP-dependent Signaling Regulates the Adipogenic Effect of *n*-6 Polyunsaturated Fatty Acids

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