

# Methionine restriction effects on 11 $\beta$ -HSD1 activity and lipogenic/lipolytic balance in F344 rat adipose tissue

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**Abstract** Methionine restriction (MR) limits age-related adiposity in Fischer 344 (F344) rats. To assess the mechanism of adiposity resistance, the effect of MR on adipose tissue (AT) 11 $\beta$ -hydroxysteroid dehydrogenase-1 (11 $\beta$ -HSD1) was examined. MR induced 11 $\beta$ -HSD1 activity in all ATs, correlating with increased tissue corticosterone. However, an inverse relationship between 11 $\beta$ -HSD1 activity and adipocyte size was observed. Because dietary restriction controls lipogenic and lipolytic rates, MR's effects on lipogenic and lipolytic enzymes were evaluated. MR increased adipose triglyceride lipase and acetyl-coenzyme A carboxylase (ACC) protein levels but induced ACC phosphorylation at serine residues that render the enzyme inactive, suggesting alterations of basal lipolysis and lipogenesis. In contrast, no changes in basal or phosphorylated hormone-sensitive lipase levels were observed. ACC-phosphorylated sites were specific for AMP-activated protein kinase (AMPK); therefore, AMPK activation was evaluated. Significant differences in AMPK $\alpha$  protein, phosphorylation, and activity levels were observed only in retroperitoneal fat from MR rats. No differences in protein kinase A phosphorylation and intracellular cAMP levels were detected. *In vitro* studies revealed increased lipid degradation and a trend toward increased lipid synthesis, suggesting the presence of a futile cycle. **In conclusion, MR disrupts the lipogenic/lipolytic balance, contributing importantly to adiposity resistance in F344 rats.**—Perrone, C. E., D. A. L. Mattocks, G. Hristopoulos, J. D. Plummer, R. A. Krajcik, and N. Orentreich. **Methionine restriction effects on 11 $\beta$ -HSD1 activity and lipogenic/lipolytic balance in F344 rat adipose tissue.** *J. Lipid Res.* 2008. 49: 12–23.

**Supplementary key words** adiposity • glucocorticoid metabolism • signal transduction pathways • 11 $\beta$ -hydroxysteroid dehydrogenase-1

Dietary restriction of the essential amino acid methionine increases longevity in Fischer 344 (F344) rats and BaLB/c1 $\times$ C57BL/6J F1 mice (1, 2). Like other dietary regimens leading to increased lifespan (3, 4), methionine restriction (MR) imposed early in life reduces body weight gain rates (5). Also, MR limits age-related increases in adi-

pose tissue (AT) mass, as indicated by reduced serum leptin and increased adiponectin levels (5), which are tightly correlated with adiposity (6, 7). Moreover, no increase in AT weight is observed in rats fed an energy-dense MR diet (1), suggesting that MR rats are resistant to obesity.

AT mass is tightly modulated by diet, energy expenditure, hormones, and tissue-specific regulators (reviewed in Refs. 8–10). Among the hormones involved in adiposity are glucocorticoids, which induce the transcription of the CCAAT enhancer binding proteins  $\alpha$ ,  $\beta$ , and  $\delta$ , leading to increased expression of the peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), an important transcription factor involved in adipogenesis and lipogenesis (11).

Glucocorticoid-mediated effects in target tissues depend on both circulating and tissue concentrations of active hormone (12), which are regulated by the 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) enzymes (13, 14). 11 $\beta$ -HSD1 is a reductase involved in the conversion of inactive glucocorticoids (cortisone and dehydrocorticosterone in humans and rodents, respectively) to their respective active forms (cortisol and corticosterone); 11 $\beta$ -HSD2 exerts the opposite effect (13, 14). Inactivation of 11 $\beta$ -HSD2, leading to increased active serum glucocorticoid levels (as in Cushing's syndrome), or overexpression of 11 $\beta$ -HSD1 in AT results in obesity (13, 15–17) and implicates glucocorticoids in the onset of central obesity and the metabolic syndrome (18, 19).

Adiposity is a complex process involving preadipocyte proliferation and differentiation as well as adipocyte hypertrophy. Moreover, adipocyte hypertrophy is determined by the balance between lipogenic and lipolytic enzymes. Insulin promotes the expression of lipogenic enzymes by activating sterol-regulatory element binding

Abbreviations: ACC, acetyl-coenzyme A carboxylase; AMPK, AMP-activated protein kinase; AT, adipose tissue; ATGL, adipose triglyceride lipase; CF, control fed; F344, Fischer 344; 11 $\beta$ -HSD, 11 $\beta$ -hydroxysteroid dehydrogenase; HSL, hormone-sensitive lipase; IGF-1, insulin-like growth factor-1; MR, methionine restriction; PKA, protein kinase A; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; SAMS peptide, substrate for AMPK-activated protein kinase; Ser, serine; SREBP-1c, sterol-regulatory element binding protein-1c; Thr172, threonine 172.

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protein-1c (SREBP-1c), inhibits the expression of lipolytic enzymes (20), and prevents lipolysis by inhibiting adenylate cyclase (reviewed in Ref. 21). In contrast to insulin, glucagon and catecholamines activate the  $\alpha_s$  subunit of G-protein, which stimulates adenylate cyclase activity and cAMP synthesis (reviewed in Ref. 22). cAMP activates protein kinase A (PKA), which phosphorylates hormone-sensitive lipase (HSL) and perilipin, allowing their association to the lipid vacuole and the catalysis of triacylglycerides into diacylglycerides (reviewed in Ref. 23).

Although HSL has been considered the key lipolytic enzyme, HSL knockout experiments revealed the presence of another lipolytic enzyme involved in the breakdown of triacylglycerides, adipose triglyceride lipase (ATGL) (24). Unlike HSL, ATGL expression is altered by nutritional regulation (20), and its inactivation increases AT mass and renders mice incapable of cold adaptation, suggesting the importance of this enzyme in the maintenance of energy balance (25).

Another mechanism controlling the lipogenic/lipolytic balance involves the allosteric regulation of acetyl-coenzyme A carboxylase (ACC). The ACC-1 isoform located in the cytosol synthesizes malonyl-CoA, which is required for fatty acid synthesis (26, 27). Malonyl-CoA produced by the ACC-2 isoform, which is located on the outer mitochondria membrane, inhibits fatty acid oxidation through inhibition of the mitochondrial carnitine palmitoyl transferase-1 (26, 27). Thus, downregulation of ACC protein levels or activity, either by allosteric ligands or by AMP-activated protein kinase (AMPK)-mediated phosphorylation, shifts lipogenesis to fatty acid oxidation (26, 27).

Although glucocorticoids have been associated extensively with increased obesity, they can also play a role in lipolysis. Administration of glucocorticoids to adult rats was reported to reduce FAS and ACC activity in AT (28). Exposure of AT to the synthetic glucocorticoid dexamethasone amplifies cAMP responses (29) and induces the phosphorylation and activation of HSL (30, 31). Moreover, ATGL activity can be induced by dexamethasone through a cAMP-independent mechanism in 3T3-L1 cells (32). Thus, glucocorticoids could potentially change AT mass by regulating signaling mechanisms that control lipogenic and lipolytic enzymes.

The objective of this study was to assess the mechanism(s) involved in MR-mediated adiposity resistance. Specifically, the correlation between 11 $\beta$ -HSD1 activity, glucocorticoid levels, and adipocyte size was evaluated. Furthermore, the effects of MR on lipogenic and lipolytic enzymes and on the activation of regulatory proteins that control the lipogenic/lipolytic balance were examined.

## METHODS

All studies were reviewed and approved by the Orentreich Foundation for the Advancement of Science, Inc., Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health guidelines for the use of animals in research laboratories.

## Materials

Purina rat chow containing 0.17% and 0.86% methionine was purchased from Dyets, Inc. (Bethlehem, PA). Insulin and adiponectin RIA kits were obtained from Linco Research/Millipore (Billerica, MA). TLC silica gel plates (0.25 mm thick with fluorescent F254 indicator) were obtained from Fisher Scientific (Pittsburgh, PA). Corticosterone and 11-hydroxycorticosterone were purchased from Steraloids, Inc. (Newport, RI). Radiolabeled [1,2,6,7-<sup>3</sup>H]corticosterone (86 mCi/mmol) was from Amersham/GE Healthcare (Piscataway, NJ). Protease and phosphatase inhibitor cocktails were from Pierce (Milwaukee, WI). Substrate for AMPK-activated protein kinase (SAMS peptide) was purchased from Upstate (Lake Placid, NY). [ $\gamma$ -<sup>32</sup>P]ATP was obtained from MP Biomedicals (Solon, OH). Type I collagenase was purchased from Worthington Biochemical Co. (Lakewood, NJ). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), except as specified otherwise.

## Animal husbandry and tissue collection

Four week old male F344 rats obtained from Taconic Farms (Germantown, NY) were maintained two rats per cage on a 12 h light/dark cycle and fed a standard control diet for 2 weeks. At 6 weeks of age, the rats were randomly assigned to control or MR diets. Food and water were provided ad libitum. After completion of the dietary regimen, the rats were anesthetized using an Euthenex Easy Anesthesia system (Palmar, PA) and blood was collected from the subclavian vein. ATs (inguinal, epididymal, retroperitoneal, and mesenteric) were then collected from control fed (CF) and MR rats, weighed, frozen immediately in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$  or fixed in 10% phosphate-buffered formalin for histological analysis. CF and MR rats were not fasted before tissue collection. Tissues were collected at 1, 2, 5, 7, 14, 21, 30, and 90 days for studies examining the effects of MR on 11 $\beta$ -HSD1 activity. Because AT weight-body weight ratios are reduced significantly by 3 months in MR, all other studies were conducted with AT samples collected at this time point.

## Serum metabolite determinations

Serum insulin, adiponectin, and corticosterone levels were measured using commercially available rat RIA kits (Linco Research/Millipore and MP Biomedicals) according to the manufacturer's instructions. Triglycerides, cholesterol, and glucose were determined using a Beckman Synchron CX5 clinical system (La Brea, CA). Free fatty acids were measured colorimetrically using a nonesterified fatty acid kit (Zen-Bio, Inc., Research Triangle Park, NC).

## 11 $\beta$ -HSD1 enzyme assay

AT was homogenized in 250 mM sucrose and 10 mM sodium phosphate buffer, pH 7.0 (4 ml/g) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 750 *g* for 30 min to precipitate the nuclear fraction and cellular debris, and the infranatant was collected and stored at  $-70^{\circ}\text{C}$  until assayed. Protein levels in the infranatant were determined using the Lowry protein assay.

11 $\beta$ -HSD1 activity was measured using a modification of the procedure reported by Lakshmi and Monder (33). Fifty micrograms of infranatant protein was added to an assay mixture consisting of 100 mM Tris-HCl, pH 8.5, 250  $\mu\text{M}$  NADP<sup>+</sup>, and 20  $\mu\text{M}$  [1,2,6,7-<sup>3</sup>H]corticosterone and preincubated at 37 $^{\circ}\text{C}$  for 10 min. The reaction was carried out for 60 min at 37 $^{\circ}\text{C}$  and stopped with ethyl acetate. After vigorous vortexing, the organic and aqueous phases were separated by centrifugation at 1,200 *g* for 5 min at 4 $^{\circ}\text{C}$ . The organic layer was collected, dried under

nitrogen, and reconstituted in 50  $\mu$ l of ethyl acetate containing 20  $\mu$ g each of unlabeled corticosterone and 11-dehydrocorticosterone. The samples were spotted onto TLC plates and developed in chloroform-methanol (92:8, v/v). Corticosterone and 11-dehydrocorticosterone spots were visualized by ultraviolet detection and scraped, and their radioactivity was measured using a Packard 1900R scintillation counter (Perkin-Elmer, Wellesley, MA). Values are expressed as means  $\pm$  SEM of four to six samples analyzed in duplicate for each tissue and time point.

### AT corticosterone measurements

Corticosterone was extracted from AT ( $n = 5$ ) using the method described by Iglesias et al. (34). Briefly, AT was homogenized in 1 mM NaOH (2 g/ml) and the homogenate was extracted with an equal volume of water-saturated ethyl acetate for 10 min at room temperature. After centrifugation at 16,000  $g$  for 10 min, the upper ethyl acetate-containing fraction was collected and dried under nitrogen at 50°C. The dried material was resuspended in 500  $\mu$ l of acetonitrile and extracted with 2 volumes of hexane. The acetonitrile (bottom) layer was dried under nitrogen and stored at -20°C. Using this method, the average corticosterone recovery, determined following the recovery of [ $^3$ H]corticosterone radioactivity in ethyl acetate fractions from AT homogenates, was 78%. Levels of corticosterone in tissue extracts were determined by RIA as specified above.

### Histology

AT fixed in 10% phosphate-buffered formalin was paraffin-embedded, sectioned at 10  $\mu$ m, and stained with hematoxylin and eosin. AT sections were photographed using a Panasonic wv-CL324 camera adapted to a Nikon Diaphot microscope. Cells ( $n = 150$ ) were counted from three randomly selected fields for each fat depot per rat ( $n = 3-4$ ), and their areas were measured using Scion Image software (version 1.32; Frederick, MD).

### Western blots

AT was homogenized in 10% sucrose, 5 mM EDTA, 6 mM MgCl<sub>2</sub>, and 50 mM Tris-HCl buffer, pH 7.2, containing phosphatase and protease inhibitors using a Polytron-type homogenizer. After homogenization, the tissue was sonicated and centrifuged at 10,000  $g$  for 10 min at 4°C. The infranatant was collected and stored at -70°C. Protein content was determined using the BCA protein assay kit (Pierce, Milwaukee, WI).

For most markers examined (except for ACC and pACC), 20  $\mu$ g of protein was electrophoresed on SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). For ACC and pACC determinations, the protein was fractionated using a 3-8% Tris-acetate gradient gel. The membranes were blocked with 5% fat-free milk in TBS-0.1% Tween and then incubated with the appropriate antibodies. Anti-ATGL (1:1,000) and anti-HSL (1:200) antibodies were purchased from Cayman Chemical (Ann Arbor, MI). Antibodies against PKA C- $\alpha$  (1:1,000), phospho-PKA C [threonine 197 (Thr197); 1:1000], ACC (1:1,000), phospho-ACC [serine 79 (Ser79); 1:1,000] and phospho-AMPK (pAMPK $\alpha$ ; 1:1,000) were from Cell Signaling Technology, Inc. (Danvers, MA). AMPK $\alpha$  antibody (1:1,000-1:2,000) was from Upstate. Anti- $\beta$ -actin antibody (1:20,000) was obtained from Sigma-Aldrich. Horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000-1:100,000) and anti-mouse IgG (1:20,000) (Bio-Rad) were used as secondary antibodies. The immunoblots were developed using the West Pico enhanced chemiluminescence kit (Pierce), except for pAMPK $\alpha$  immunoblots, which were developed with the West Femto detection kit (Pierce). The density of bands on X-ray films was analyzed using the Un-Scan-it gel digitizing software (Silk Scientific, Inc., Orem, UT).

Protein levels were standardized against  $\beta$ -actin levels, and the data are expressed as means  $\pm$  SEM of four to five individual samples.

### AMPK assay

AMPK activity was determined using a modification of the protocol reported by Stapleton et al. (35). AT was homogenized at 4°C in lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM DTT, 1 mM benzamide, 200  $\mu$ M PMSF, and a protease inhibitor cocktail (Pierce). The homogenate was centrifuged at 12,000  $g$  for 20 min at 4°C and the supernatant was collected. Considering reports by Park et al. (36) showing a lack of activity in immunopurified AMPK from AT, AMPK assays were conducted using precipitated protein. Proteins were precipitated from the 12,000  $g$  supernatant for 30 min at 4°C with 35% (w/v) ammonium sulfate followed by centrifugation at 45,000  $g$  for 30 min at 4°C in a Beckman L8-C1 centrifuge. The protein pellet was resuspended in lysis buffer and stored at -70°C until assayed. Protein content was determined using the BCA protein assay kit.

AMPK activity was followed in an assay mixture consisting of 40 mM HEPES, pH 7.0, 80 mM NaCl, 8% glycerol, 0.8 mM DTT, 5 mM MgCl<sub>2</sub>, 0.2 mM AMP, 0.2 mM ATP, 0.2 mM SAMS peptide, and precipitated protein in a 20  $\mu$ l final volume. For each sample, reactions were conducted in duplicate and in the presence or absence of substrate. The assay mixture was incubated at 37°C for 10 min, and the reaction was stopped by spotting an aliquot of assay mixture onto p81 Whatman filter paper and placing the filter paper in 1% phosphoric acid. The filters were rinsed six times in 1% phosphoric acid (30 min rinses) and once in acetone for 5 min. After the acetone rinse, the filters were air-dried and placed in scintillation counter fluid.  $^{32}$ P incorporation into the SAMS peptide was measured using a Packard 1900R scintillation counter (Perkin-Elmer).

### cAMP measurements

Frozen AT ( $n = 5$ ) was homogenized in 5 volumes (ml/g tissue) of ice-cold 5% TCA using a Polytron-type homogenizer. The homogenate was centrifuged at 1,500  $g$  for 10 min, and the supernatant was collected and extracted three times with water-saturated ether. The ether fraction was discarded and the residual ether in the aqueous fraction was removed by heating at 70°C for 10 min. cAMP in the aqueous fraction was measured by enzyme immunoassay (Cayman Chemical) according to the manufacturer's procedure.

### Lipid synthesis and degradation studies

Lipid synthesis and degradation studies were conducted using isolated epididymal mature adipocytes. AT ( $n = 4$ ) from CF and MR rats was rinsed with modified Krebs-Ringer bicarbonate buffer, pH 7.4, containing 4% NEFA-free BSA and 3 mM glucose, then minced and digested in collagenase buffer consisting of 50 mM HEPES, 100 mM NaCl, 5.5 mM D-glucose, 5.4 mM KCl, 15 mM NaHCO<sub>3</sub>, 6 mM CaCl<sub>2</sub>, 4% NEFA-free BSA, and 400 U/ml collagenase type I at 37°C and 5% CO<sub>2</sub> atmosphere. The cells were filtered through 200  $\mu$ m nylon mesh, and mature adipocytes were separated from preadipocytes by buoyancy. The infranatant containing preadipocytes was discarded, and the mature adipocytes were rinsed three times with modified Krebs-Ringer bicarbonate buffer and counted.

Lipolysis was determined by evaluating fatty acid and glycerol release into the medium (37). Mature adipocytes were incubated at 37°C and 5% CO<sub>2</sub> for 1 h in modified Krebs-Ringer bicarbonate buffer containing 1 U/ml adenosine deaminase in the presence or absence of 10<sup>-5</sup> M isoproterenol. After the incubation period, the

TABLE 1. Serum chemistry profiles in CF and MR rats

Parameter	1 Month CF	1 Month MR	3 Months CF	3 Months MR
Insulin (ng/ml)	1.48 ± 0.26	0.91 ± 0.18 <sup>a</sup>	0.92 ± 0.26	0.43 ± 0.07
Adiponectin (ng/ml)	5.18 ± 0.26	9.92 ± 0.40 <sup>b</sup>	3.71 ± 0.35	12.35 ± 0.46 <sup>b</sup>
Corticosterone (ng/ml)	367 ± 110	597 ± 77	252 ± 64	301 ± 86
Glucose (mg/dl)	188 ± 13	175 ± 21	207 ± 5	163 ± 3 <sup>b</sup>
Triglycerides (mg/dl)	149 ± 31	47 ± 16	106 ± 12.7	32 ± 2.9 <sup>c</sup>
Free fatty acids (mEq/dl)	0.2 ± 0.1	0.3 ± 0.2	14 ± 1	16 ± 2
Cholesterol (mg/dl)	47 ± 4	43 ± 4	46 ± 1.6	40 ± 0.9 <sup>d</sup>

CF, control fed; MR, methionine restriction. Values are expressed as means ± SEM of five to seven samples and analyzed by ANOVA.

<sup>a</sup>*P* = 0.041.

<sup>b</sup>*P* < 0.001.

<sup>c</sup>*P* = 0.002.

<sup>d</sup>*P* = 0.026.

adipocyte suspensions were placed on ice and immediately centrifuged at 3,500 *g* for 5 min at 4°C. The infranatants were collected and stored at -20°C until analyzed. Fatty acid released into the medium was measured using a NEFA detection kit (Zen-Bio, Inc.). Glycerol content was measured enzymatically using Sigma's free glycerol reagent. Samples were analyzed in triplicate.

Lipid synthesis was determined by assessing the incorporation of [2-<sup>14</sup>C]acetate into fatty acids (38). Adipocytes were incubated for 1 h at 37°C and 5% CO<sub>2</sub> in modified Krebs-Ringer medium containing 10 mM acetate and 2 μCi/ml [2-<sup>14</sup>C]acetate in the presence or absence of 10<sup>-9</sup> M insulin in a 500 μl final volume. The reaction was stopped with 200 μl of 8 N H<sub>2</sub>SO<sub>4</sub>, and fatty acids were extracted with 2.5 ml of Dole's reagent (39). After incubating the extraction mixture for 5 min at room temperature, 1 ml of water and 1.5 ml of heptane were added to the Dole's reagent to shift the mixture to a two-phase system. An aliquot of the upper

phase was collected and [2-<sup>14</sup>C]acetate incorporation into fatty acids was measured with a Packard 1900R scintillation counter (Perkin-Elmer). Samples were analyzed in triplicate.

### Data analysis

The data are expressed as means ± SEM and were analyzed by ANOVA using SigmaStat software (Systat, San Jose, CA).

## RESULTS

### Serum chemistry profile of MR rats

Long-term MR induces hormonal and metabolite changes that correlate with decreased adiposity and improved insulin

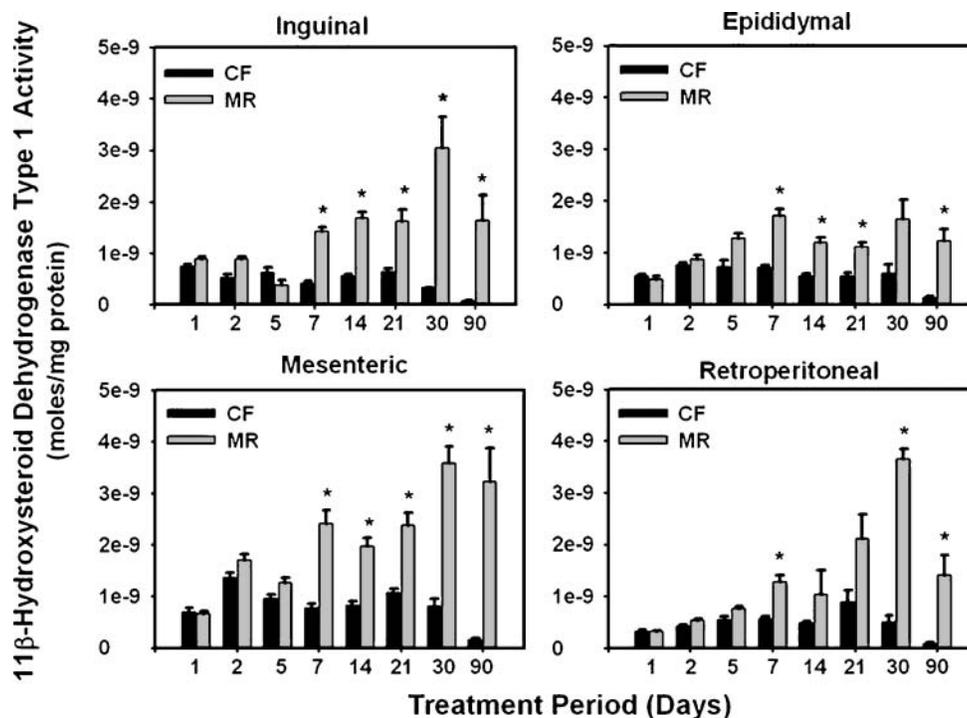


Fig. 1. 11β-Hydroxysteroid dehydrogenase-1 (11β-HSD1) activity in adipose tissue (AT) from control fed (CF) and methionine restriction (MR) Fischer 344 rats. The rats were fed ad libitum, and ATs were collected and frozen at various time points. The tissues were homogenized, and 11β-HSD1 activity was determined as described in Methods. Each bar represents the mean ± SEM of four to six samples per time point and treatment group tested in duplicate and analyzed by ANOVA. \* *P* < 0.05.

sensitivity in F344 rats (5); therefore, we examined early MR effects on serum chemistry. Although 1 month of MR caused no significant changes in serum metabolites, insulin levels were decreased significantly (39%) and adiponectin levels were increased significantly (48%) in MR rats compared with CF rats (Table 1). By 3 months of MR, glucose and triglycerides were reduced significantly (21% and 69%, respectively) (Table 1). A marginal yet significant decrease in cholesterol was also observed in 3 month MR rats. Insulin levels were also reduced in 3 month MR rats but were not significantly different from insulin levels in CF rats (Table 1). Finally, MR caused no significant changes in serum corticosterone levels at 1 and 3 months (Table 1).

### Effects of MR on 11 $\beta$ -HSD1 activity

To determine whether the MR-mediated adiposity resistance correlates with changes in AT 11 $\beta$ -HSD1 activity, enzyme assays were conducted. 11 $\beta$ -HSD1 activity was increased in fat depots from MR rats as early as 7 days on the diet and remained high at 1 and 3 months on MR (Fig. 1). To verify the MR effects on 11 $\beta$ -HSD1, corticosterone levels were measured in AT from 1 and 3 month CF and MR rats. AT corticosterone was increased at 1 and 3 months of MR, corresponding with increased 11 $\beta$ -HSD1 activity (Fig. 2).

Corticosterone is also metabolized in liver; therefore, the effect of MR on liver 11 $\beta$ -HSD1 was examined. In contrast to AT, no changes in liver 11 $\beta$ -HSD1 activity were observed in MR rats (Fig. 3), suggesting that the MR effects were AT-specific.

### Reduced mature adipocyte area in fat depots from MR rats

Induction of 11 $\beta$ -HSD1 activity and increased tissue corticosterone levels have been associated with increased adipogenesis and lipogenesis, in contrast to observations on adiposity resistance by MR. Because corticosterone can also induce lipolysis (29, 40, 41), morphometric analyses were conducted to assess whether there were histological differences in ATs from CF and MR rats. Hematoxylin and eosin staining revealed that mature adipocytes from 1 and 3 month MR rats were significantly smaller than adipocytes from CF rats (Fig. 4A, B). On average, 46, 44, 23, and 38% reductions in the cross-sectional areas of inguinal, epididymal, mesenteric, and retroperitoneal adipocytes, respectively, were observed at 3 months in MR (Fig. 4B, C). These results suggest that MR could induce changes in the lipogenic/lipolytic balance.

### MR effects on the expression and phosphorylation levels of lipogenic and lipolytic enzymes

Western blotting analysis was conducted to evaluate whether decreased mature adipocyte cross-sectional area in response to MR correlated with an increased expression of lipolytic enzymes. No significant changes in basal levels of ATGL and HSL were observed at 1 month on the MR diet (data not shown). However, by 3 months, a significant increase in ATGL levels was observed (Fig. 5A), suggesting upregulation of basal lipolysis. In contrast, no change in

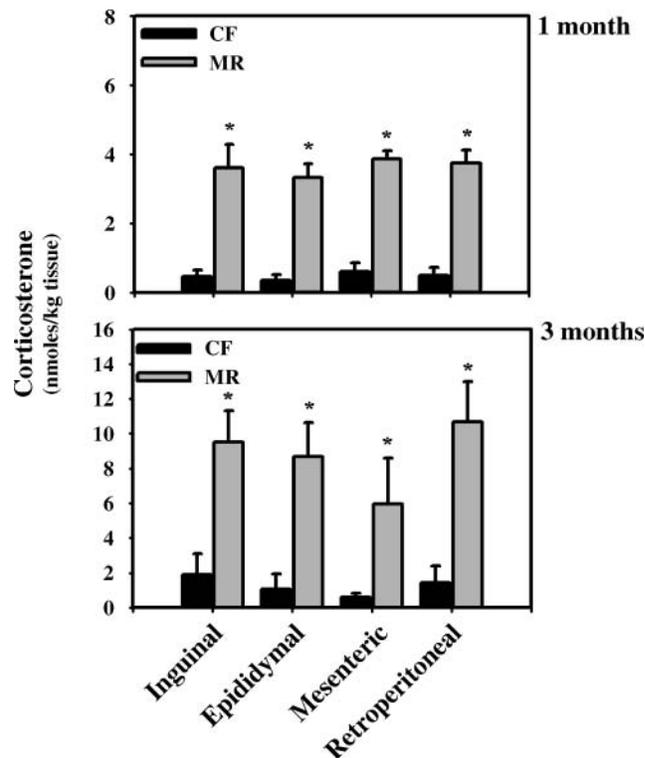


Fig. 2. Corticosterone levels in ATs from CF and MR rats. Tissues were collected from 1 and 3 month CF and MR rats, and corticosterone levels were measured from AT extracts by enzyme immunoassay as described in Methods. Values are expressed as means  $\pm$  SEM of five samples per group and analyzed by ANOVA. \*  $P < 0.05$ .

HSL expression was observed in AT from 3 month MR rats (Fig. 5B). Because HSL activity can be upregulated by phosphorylation (23), the potential phosphorylation of HSL after MR at Ser563 (site for PKA phosphorylation) or Ser565 (site for AMPK phosphorylation) was evaluated. Phosphorylation of HSL Ser563 was not detected by

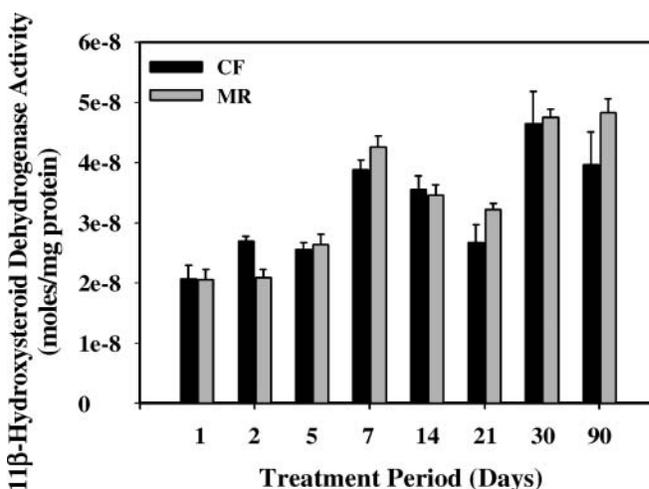
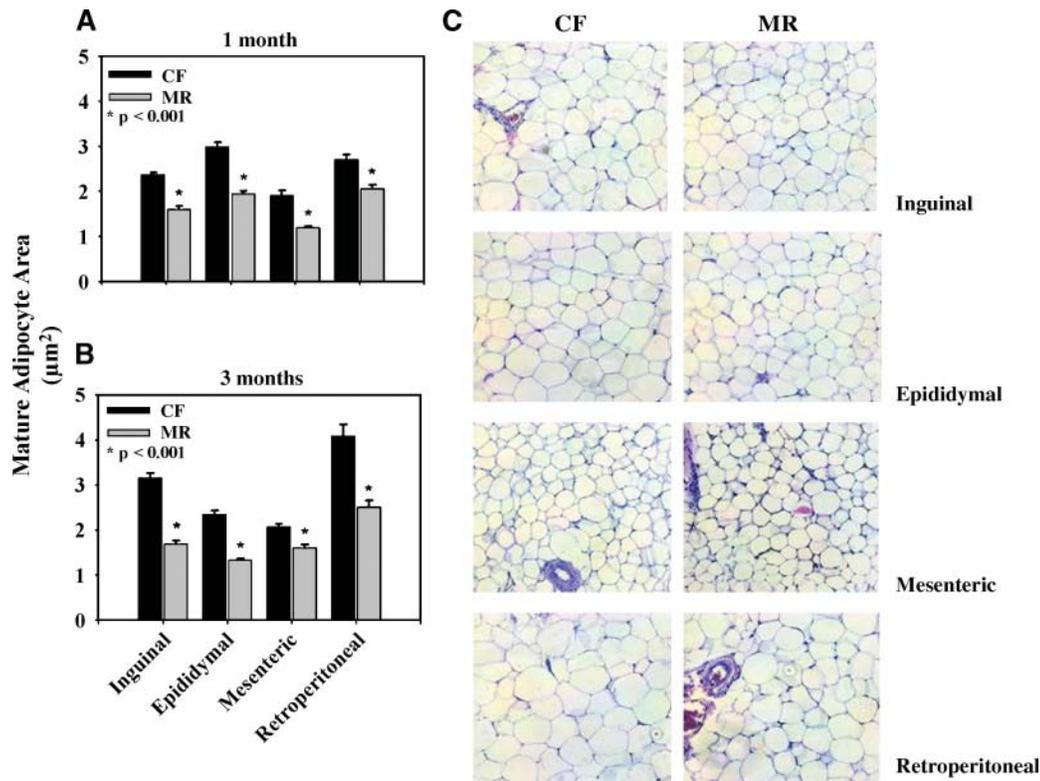


Fig. 3. 11 $\beta$ -HSD1 activity in liver from CF and MR rats. Liver tissue was homogenized, and 11 $\beta$ -HSD1 activity was determined as described in Methods. The data are presented as means  $\pm$  SEM of six samples per time point and treatment group tested in duplicate and analyzed by ANOVA.



**Fig. 4.** Morphometric analysis of ATs from 1 month (A) and 3 month (B, C) CF and MR rats. Inguinal, epididymal, mesenteric, and retroperitoneal ATs were paraffin-embedded, sectioned at 10  $\mu\text{m}$ , and stained with hematoxylin and eosin. Adipocytes were counted from three randomly selected fields for each fat depot per rat, and their areas were measured using Scion Image software as described in Methods. Values are expressed as means  $\pm$  SEM.

Western blotting. In contrast, a trend showing increased HSL Ser565 phosphorylation in AT from MR rats was observed, although HSL Ser565 phosphorylation was not significantly different from that in CF rats (Fig. 5C).

Another key enzyme involved in the maintenance of the lipogenic/lipolytic balance is ACC (27). ACC is inactivated by phosphorylation at Ser residues 79, 1,200, and 1,215 after signaling of inhibitory hormones. Also, during fasting, lipolysis is stimulated after the degradation of ACC by ubiquitination promoted by the pseudokinase Tribbles 3 protein (26). Therefore, ACC protein and phosphorylation (using a phospho-specific antibody for Ser79) levels were examined in ATs from CF and MR rats. In contrast to fasting and caloric restriction, in which ACC levels are decreased (42), MR significantly increased levels of both ACC-1 and ACC-2 isoforms in AT, except for mesenteric fat depots (Fig. 5D). Moreover, both ACC isoforms were significantly phosphorylated at the Ser79, except for mesenteric AT (Fig. 5E).

#### MR effects on AMPK, PKA, and cAMP levels in AT

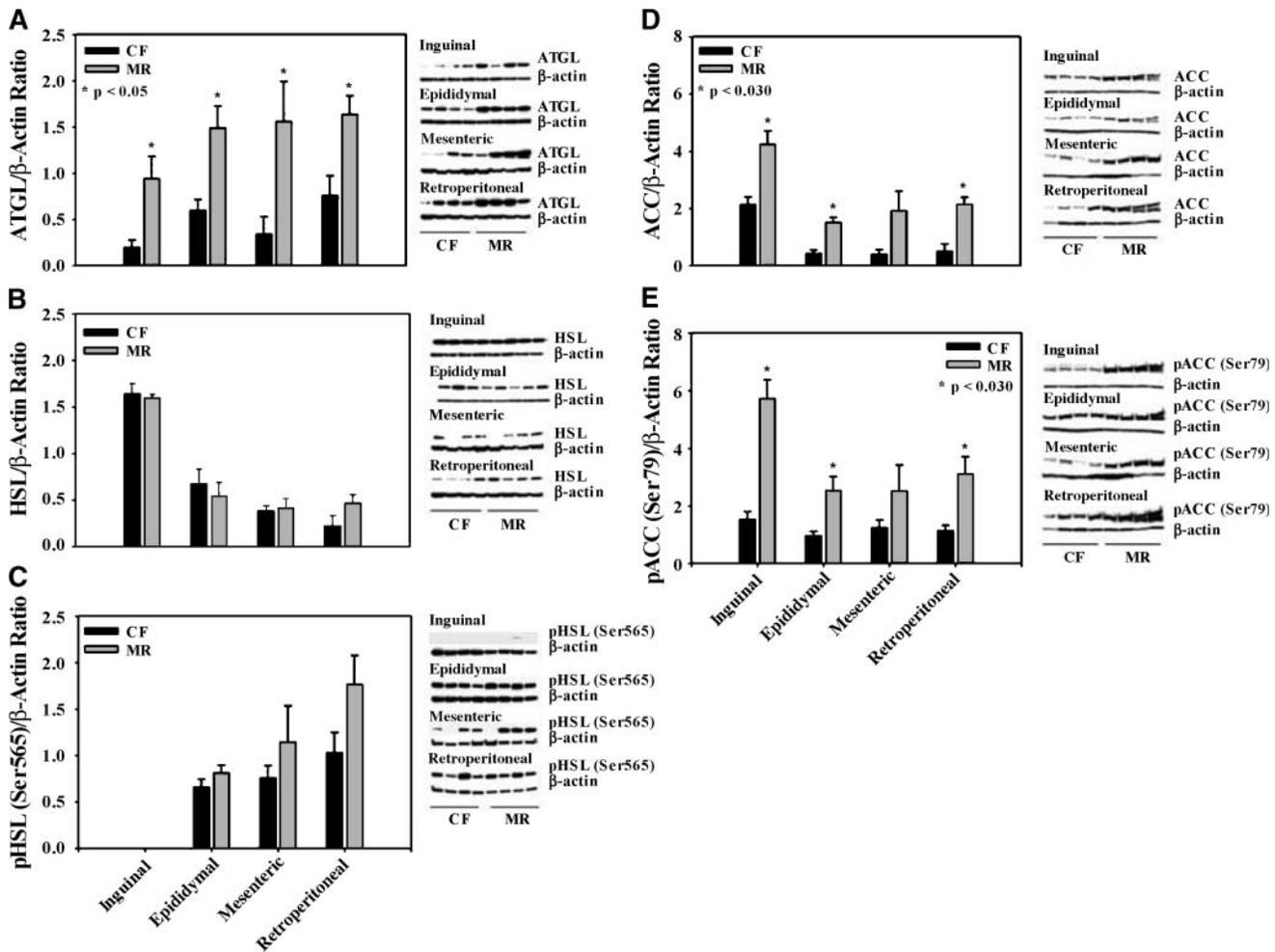
AT lipolysis is controlled by hormonal and neuronal factors that upregulate the synthesis of cAMP and the downstream phosphorylation of PKA or that activate AMPK (reviewed in Ref. 43). AMPK activation by Thr172 phosphorylation was first examined considering that AMPK phosphorylates and inactivates ACC (23, 26, 28). Although

there is a trend showing decreased basal AMPK levels, no significant differences were observed in ATs from CF and MR rats (Fig. 6A). Moreover, no significant changes in AMPK Thr172 phosphorylation were observed in AT from MR rats, except for retroperitoneal fat (Fig. 6B). Considering that MR reduced basal APMK protein levels, pAMPK/AMPK $\alpha$  ratios were estimated. No significant differences in pAMPK/AMPK $\alpha$  ratios were observed, except for retroperitoneal fat, in which it was increased (data not shown). To further confirm the MR effects on AMPK activation, we conducted AMPK enzyme assays. Corresponding with the Western blot data, no significant differences in AMPK activity were observed in AT from MR rats, except for retroperitoneal fat (Table 2).

The classical lipolytic pathway involves G-protein activation and cAMP synthesis, which activates PKA, resulting in the downstream phosphorylation of HSL. Therefore, cAMP and PKA phosphorylation levels were assessed in AT extracts. No changes in cAMP levels were detected in MR fat depots (Fig. 7A), suggesting that MR had no effect on PKA activity. These results agreed with Western blotting showing no changes in PKA C- $\alpha$  protein and phosphorylation levels (Fig. 7B, C).

#### Lipid metabolism in AT from MR rats

Considering the observed MR effects on ATGL expression levels suggesting increased lipid degradation in ATs



**Fig. 5.** Western blots for adipose triglyceride lipase (ATGL; A), hormone-sensitive lipase (HSL; B), phospho-HSL (pHSL; C), acetyl-coenzyme A carboxylase (ACC; D), and phospho-ACC (pACC; E) from CF and MR rat ATs. Proteins were fractionated by gel electrophoresis, electroblotted onto polyvinylidene difluoride membranes, and probed for specific antibodies as described in Methods. Relative protein concentrations normalized over  $\beta$ -actin were determined using the Un-Scan-it gel digitizing software. Values are expressed as means  $\pm$  SEM ( $n = 4-5$ ) and analyzed by ANOVA. Ser, serine.

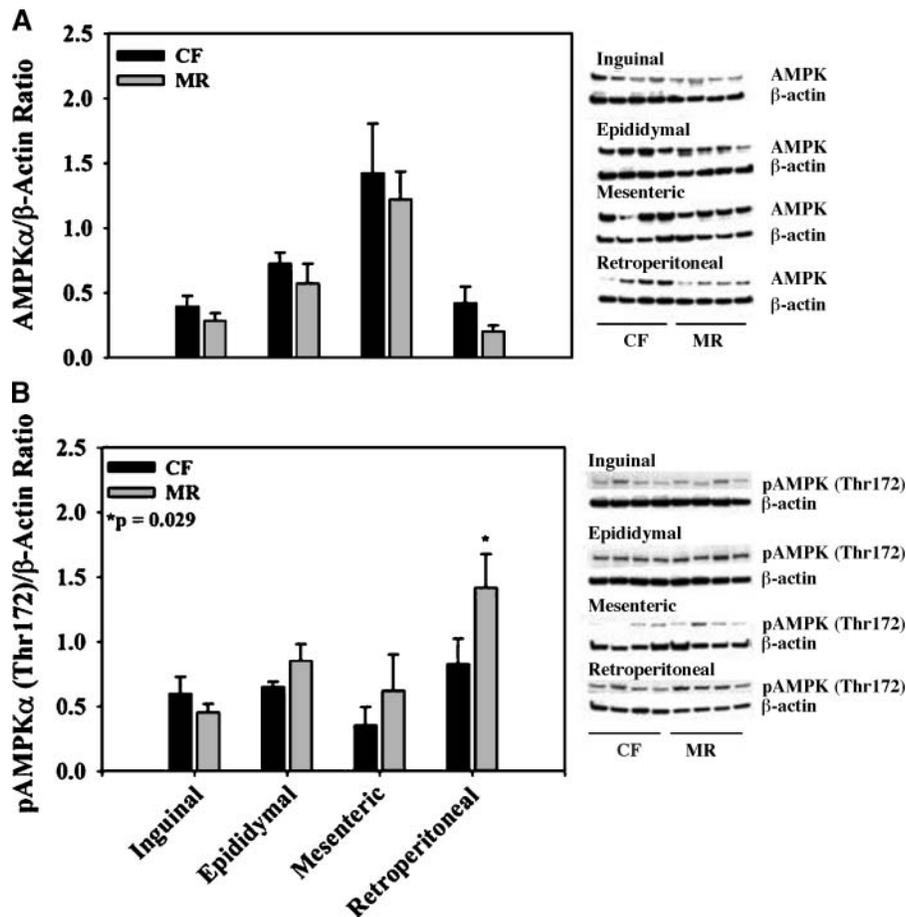
from MR rats, lipolysis studies in epididymal mature adipocytes were conducted. Epididymal tissue was chosen given that all ATs from MR rats show similar ATGL protein changes. On average, mature adipocytes from CF rats released 18.6 nM NEFA/ $10^6$  cells into the medium (Fig. 8A). A significant 58% increase in fatty acid release was observed in isoproterenol-treated adipocytes from CF rats (Fig. 8A). Corresponding with the MR-mediated effects on ATGL expression, basal fatty acid release from MR rat adipocytes was 3-fold greater than that observed from CF rat adipocytes (Fig. 8A). Moreover, fatty acid release from MR rat adipocytes was further induced by isoproterenol treatment (Fig. 8A). Corresponding with the fatty acid release data, a trend toward an increase in glycerol release from MR adipocytes was observed (Fig. 8B). The glycerol release data from MR rat adipocytes correlate with the findings that HSL protein and phosphorylation levels were not changed significantly by MR. Increased ACC phosphorylation in MR rats suggested decreased lipid synthesis; therefore, MR's effect on lipogenesis was also examined in vitro. Contrary to our

hypothesis, a trend toward an increase in fatty acid synthesis was observed in mature adipocytes from MR rats (Fig. 8C). These results suggest the possible presence of a futile cycle in ATs from MR rats.

## DISCUSSION

This study revealed that MR increases 11 $\beta$ -HSD1 activity, as confirmed by high AT corticosterone levels. Despite ATs from MR rats producing high levels of corticosterone, MR rat mature adipocytes were smaller than those from CF rats. The decreased adipocyte size correlated with increased ATGL expression and ACC phosphorylation, suggesting induction of lipid degradation and suppression of fatty acid synthesis. However, in vitro studies revealed increased lipolysis and a trend toward increased lipogenesis in ATs from MR rats, suggesting the presence of a lipid-futile cycle.

Although the mechanism of MR-induced glucocorticoid synthesis is unknown, it might be attributable to reduced



**Fig. 6.** AMP-activated protein kinase (AMPK $\alpha$ ; A) and phospho-AMPK (pAMPK $\alpha$ ; B) levels in ATs from CF and MR rats. Proteins were fractionated by 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. Western blots were analyzed using the Un-Scan-it gel digitizing software. Relative protein levels are expressed as means  $\pm$  SEM ( $n = 4-5$ ) and analyzed by ANOVA. Thr, threonine.

insulin-like growth factor-1 (IGF-1) levels observed in MR rats (2, 5). Studies conducted by Moore et al. (44) showed that acromegalic patients with increased serum levels of growth hormone and IGF-1 have low 11 $\beta$ -HSD1 activity. The same investigators confirmed that IGF-1 inhibits 11 $\beta$ -HSD1 activity (but not 11 $\beta$ -HSD2) in an in vitro system using omental adipose stromal cells transfected with human 11 $\beta$ -HSD1 and 11 $\beta$ -HSD2 genes (44). Similar observations were drawn from a study of patients with hypothyroidism showing that growth hormone replacement decreased the ratio of 11-hydroxyl/11-oxo-cortisol

metabolites, suggesting the inhibition of 11 $\beta$ -HSD1 by growth hormone/IGF-1 (45).

Despite extensive evidence demonstrating that high AT 11 $\beta$ -HSD1 activity correlates positively with visceral adiposity and metabolic syndrome in both humans and laboratory animals (16, 18, 46–49), this study showed an inverse relationship between 11 $\beta$ -HSD1 activity levels and adipocyte size. In addition to inducing preadipocyte differentiation into mature adipocytes (50, 51), glucocorticoids have a dual role in lipogenesis and lipolysis (29, 40, 41). In fact, ATGL expression is decreased in the *ob/ob* and *db/db* obese mouse models and can be induced by fasting or by supplementation with the synthetic glucocorticoid dexamethasone (32). Glucocorticoids were also reported to decrease the activity of the lipogenic enzymes FAS and ACC (28). Western blot analysis showed significant increases in ATGL protein and ACC phosphorylation levels by MR, suggesting the possible contribution of glucocorticoids on the lipogenic/lipolytic balance. Decreased insulin levels can also mediate ATGL upregulation (20); therefore, it cannot be concluded that induced ATGL expression is a response to increased active tissue glucocorticoid levels, because MR rats have lower plasma insulin levels than CF rats. Moreover, 11 $\beta$ -HSD1 induction is ob-

**TABLE 2.** AMPK activity in AT from 3 month CF and MR rats

AT	AMPK Activity	
	CF	MR
	<i>nmol/min/mg protein</i>	
Inguinal	6.31 $\pm$ 1.19	5.07 $\pm$ 1.05
Epididymal	18.20 $\pm$ 6.59	19 $\pm$ 3.92
Mesenteric	2.26 $\pm$ 1.19	3.62 $\pm$ 0.99
Retroperitoneal	0.29 $\pm$ 0.09	0.87 $\pm$ 0.12 <sup>a</sup>

AMPK, AMP-activated protein kinase; AT, adipose tissue. Values are expressed as means  $\pm$  SEM of five samples and analyzed by ANOVA.  
<sup>a</sup>  $P = 0.015$ .

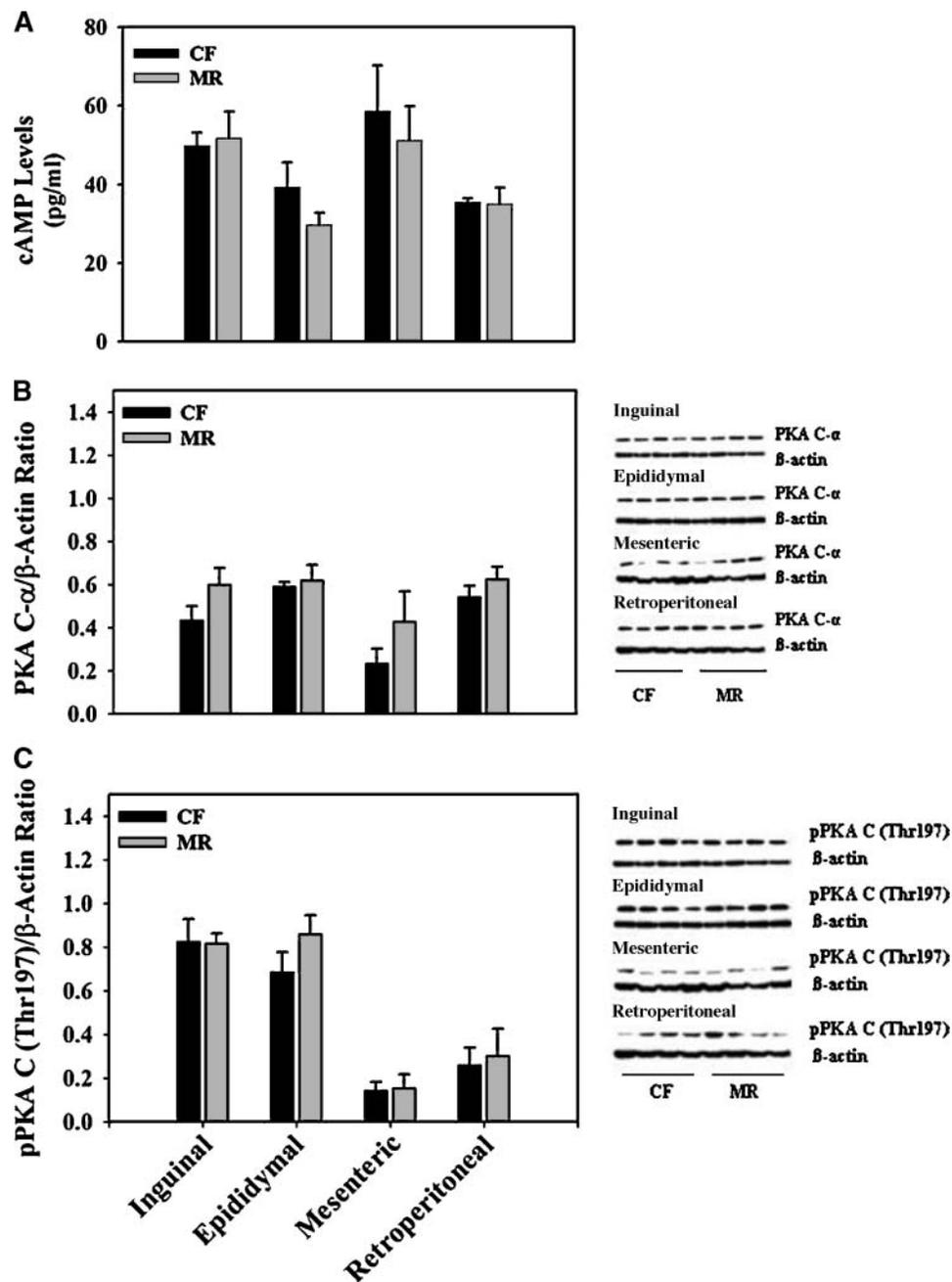


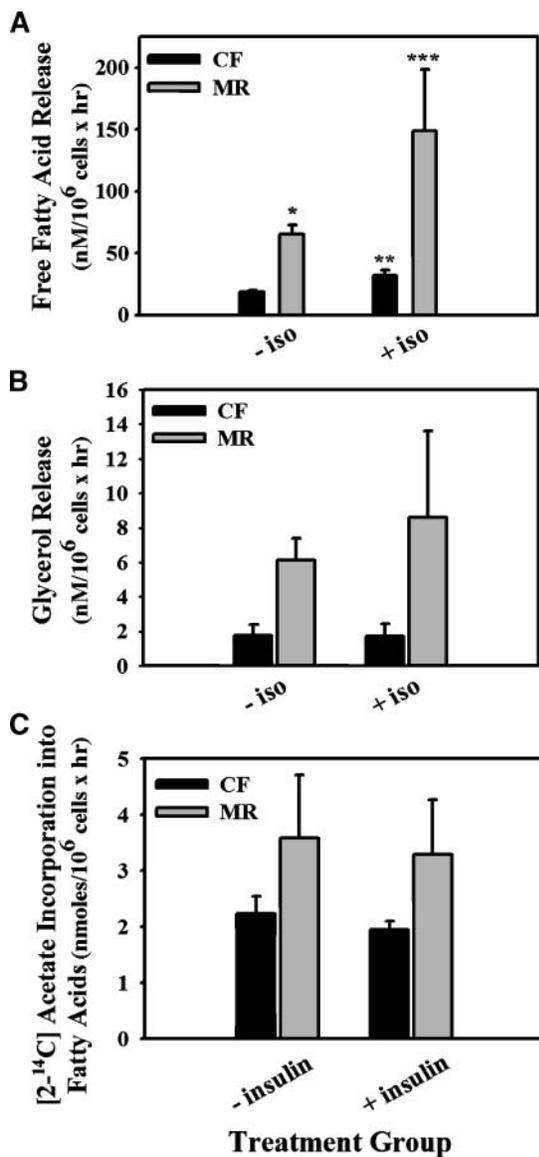
Fig. 7. cAMP (A), protein kinase A (PKA) C- $\alpha$  (B), and pPKA C (C) in ATs from CF and MR rats. cAMP was measured by enzyme immunoassay. Levels of PKA C- $\alpha$  and pPKA C were measured by Western blotting as described in Methods. Relative protein and cAMP levels are expressed as means  $\pm$  SEM ( $n = 4-5$ ) and analyzed by ANOVA.

served as early as 1 week on MR, whereas changes in lipogenic and lipolytic enzymes occur after 1 month on the dietary restriction. This suggests that other physiological changes may be required to alter the lipogenic/lipolytic balance in MR rats.

Decreased cross-sectional adipocyte area might be a response to other factors (e.g., AT-derived adiponectin). Adiponectin can activate AMPK, leading to the phosphorylation and inhibition of ACC as well as the induction of fatty acid oxidation in C2C12 myotubes (52). Chronic over-expression of adiponectin in mouse AT reduces fat pad

weight as a function of decreased preadipocyte differentiation and adipocyte diameter as well as increased energy expenditure, marked by decreased FAS expression and increased uncoupling protein 2 (53). Considering the known effects of adiponectin on fatty acid storage and oxidation in light of the ability of MR to increase serum adiponectin levels, it is possible that adiponectin contributes importantly to the reduced adipocyte cross-sectional area observed in MR rats.

There is also increasing evidence that amino acids can function as signaling molecules controlling glucose trans-



**Fig. 8.** Lipid synthesis and degradation in AT from CF and MR rats. Mature adipocytes were isolated by collagenase digestion as described in Methods. A, B: Lipid degradation was determined by measuring NEFA (A) and glycerol (B) release from the adipocytes in the presence or absence of isoproterenol (iso). C: Lipid synthesis was assessed after [<sup>2-<sup>14</sup>C</sup>]acetate incorporation into fatty acids. Values are expressed as means  $\pm$  SEM ( $n = 4$ ) and analyzed by ANOVA. \*  $P < 0.001$ , CF -iso versus MR -iso; \*\*  $P = 0.031$ , CF -iso versus CF +iso; \*\*\*  $P < 0.05$ , CF +iso versus MR +iso and MR -iso versus MR +iso.

port and homeostasis, gene expression, and protein translation through the modulation of the mammalian target of rapamycin pathway (reviewed in Ref. 54). Glutamate dephosphorylates and activates ACC-1 in Wistar rat liver and AT, suggesting a role for amino acids in lipid metabolism and homeostasis (55). More recently, leucine deprivation was shown to repress lipid synthesis in mouse liver by reducing SREBP-1c and PPAR $\gamma$  expression through the activation of the control nondepressible 2 kinase (56). Therefore, decreased methionine levels may

have direct effects on the lipolytic/lipogenic balance. Our MR studies show that restriction of an essential amino acid not only changes the expression of lipogenic and lipolytic enzymes but also induces posttranslational modifications of lipogenic enzymes, such as ACC phosphorylation.

ACC activity is under the control of insulin and catabolic hormones via phosphorylation at different sites (27). Hormonal inactivation of ACC is mediated not only by cAMP but also by phosphorylation of Ser residues 79, 1,200, and 1,215, possibly by AMPK (27). Although basal levels of ACC-1 and ACC-2 were increased in ATs by MR, the two isoforms were phosphorylated at Ser79, as determined by a phospho-specific antibody, implicating the possible inactivation of this enzyme.

ACC phosphorylation at Ser79 has been attributed to AMPK (57–59). The allosteric binding of AMP to the AMPK $\gamma$  subunit facilitates the phosphorylation of Thr172 in the  $\alpha$  subunit, thereby leading to AMPK activation (60). Although ATs from MR rats had increased pACC levels, no significant increases in pAMPK $\alpha$  and AMPK activities were detected, except for retroperitoneal fat. This is not the only report unable to demonstrate AMPK activation during chronic dietary restriction. In fact, failure to show an induction of AMPK activity has also been reported for metabolic adaptations of mice to fasting or to chronic caloric restriction (42). This could be explained by the transient nature of AMPK phosphorylation.

Increased ATGL protein levels and ACC phosphorylation suggested that increased fatty acid oxidation and reduced fatty acid synthesis could contribute to decreased adiposity in MR rats; therefore, lipid synthesis and degradation studies were conducted to confirm this hypothesis. In vitro studies using mature adipocytes confirmed an increase in basal lipolysis correlating with the MR-mediated induction of ATGL protein levels. In contrast, a trend toward an increase of lipogenesis in ATs from MR rats contradicted the hypothesis of MR-mediated ACC phosphorylation and inactivation. One possibility is that the ACC phosphorylation state could have been altered during the isolation of mature adipocytes and their incubation in modified Krebs bicarbonate buffer, which contained glucose. Another possibility is that MR induced changes not only in ACC phosphorylation but also in basal ACC protein levels, which could contribute to increased fatty acid synthesis.

The induction of lipid synthesis and degradation suggests that a lipid-futile cycle in ATs from MR rats may be involved in adiposity resistance. In fact, mature adipocytes possess the ability to use products of lipolysis to resynthesize lipids through the induction of glycerol kinase after the activation of the nuclear receptor PPAR $\gamma$  (61). PPAR $\gamma$  activation also induces the expression of ATGL and monoacylglyceride lipase but not HSL in ATs, leading to increased basal lipolysis, and exacerbates adrenergic-mediated lipolysis (62). Glucocorticoids such as dexamethasone activate PPAR $\gamma$  and have been reported to induce ATGL expression (11, 32). It is possible that increased corticosterone synthesis in ATs from MR rats may upregulate ATGL gene expression, thereby driving the lipid-futile cycle.

In conclusion, changes in the lipogenic/lipolytic balance leading to a lipid-futile cycle appear to play important roles in MR-mediated adiposity resistance. MR-mediated changes in the lipogenic/lipolytic balance might result from increased AT corticosterone and adiponectin levels as well as decreased insulin signaling, IGF-1, or methionine itself. This study provides insight into the intracellular responses of AT to MR, but further investigation is required to establish how the physiological changes induced by MR integrate, resulting in adiposity resistance. 

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