The Effects of Hormone Replacement Therapy on Carbohydrate Metabolism and Cardiovascular Risk Factors in Surgically Postmenopausal Cynomolgus Monkeys

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Controversy exists regarding the effects of estrogen and estrogen/progestin replacement therapies on glucose tolerance and insulin resistance. Also unknown are whether changes in glucose tolerance and insulin resistance with hormone therapy affect arterial glycation and atherosclerosis. We studied ovariectomized female monkeys fed a lipid-lowering diet and given either no hormone replacement therapy (n = 25) or conjugated equine estrogens (CEE) alone (n = 22) or combined with medroxyprogesterone acetate ([MPA] n = 21) for 30 months. Monkeys receiving combined hormone replacement had significantly higher fasting glucose and insulin levels and higher insulin responses to a glucose challenge compared with controls or those given estrogen alone. Monkeys given estrogen-only therapy had lower body weights, lower measures of abdominal adiposity, and decreased serum androgen concentrations. However, due to the effective dietary lipid decrease, there was no additional effect of hormone treatment on atherosclerosis. Also, there was no correlation between either arterial glycation or insulin levels and atherosclerosis extent. Thus, although there were adverse effects of combined hormone replacement therapy on carbohydrate metabolism, we were unable to determine whether these effects altered the extent of atherosclerosis.

CORONARY HEART DISEASE (CHD) is the leading cause of death in women in Western societies. Postmenopausal estrogen replacement therapy reduces the risk of CHD by about 50%.[1,2] Some of this beneficial effect may be mediated by estrogen-induced increases in high-density lipoprotein cholesterol and decreases in low-density lipoprotein cholesterol concentrations, but the major effect (50% to 75%) appears to be independent of plasma lipid and lipoprotein concentrations.[2] We have shown that estrogens may have direct effects on the artery[3,4] that may account for some of the non–lipid-mediated beneficial effect. Insulin resistance, carbohydrate metabolism, and regional adiposity also are risk factors for CHD[5-10] and may be additional mechanisms by which sex hormones influence CHD in women.

Although insulin resistance has been shown to be a significant cardiovascular risk factor in women,[5-8] conflicting reports exist in the literature regarding estrogens and insulin concentrations or sensitivity (for review, see Skouby[11]). A progestin is usually added to the estrogen replacement therapy for postmenopausal women to reduce the risk of endometrial cancer, but this may result in increased insulin resistance.[12-17] We have reported that progestins alone or in combination with estrogens can induce insulin resistance.[18] Whether hyperinsulinemia resulting from insulin resistance increases the risk of cardiovascular disease in postmenopausal women remains unknown.[19]

The central distribution of body fat has been recognized as an independent predictor of cardiovascular disease in women.[10] An increase in central adiposity adversely affects carbohydrate metabolism and insulin resistance and is associated with elevated androgen levels.[9] The postmenopausal shift from a peripheral to a more central fat distribution may be responsible for some of the increased risk for CHD in postmenopausal women.[20] Furthermore, hormone replacement therapy has been associated with decreased abdominal fat.[20]

Carbohydrate intolerance and hyperglycemia have also been found to increase the risk of CHD.[21] Increased vascular tissue glycation secondary to hyperglycemia is one postulated mechanism that may contribute to this process. The glycation mechanism involves nonenzymatic reactions that occur between glucose and proteins or nucleic acid amino groups to form labile Schiff bases, which rearrange to form stable but chemically reversible Amadori products.[22,23] With time, Amadori products dehydrate, rearrange, and form irreversible structures referred to as advanced glycation end products (AGE).[24] AGE moieties are brown fluorescent chromophores that can cross-link proteins,[25] and vascular accumulation of AGE may exacerbate atherogenesis.[25,26]

We have found the cynomolgus monkey to be an excellent animal model for studies of both cardiovascular disease[27] and carbohydrate metabolism,[18,28] and in female monkeys, estrogen replacement therapy reduces atherosclerosis progression by approximately 50%.[27] This study was designed to examine the effects of hormone replacement therapy and decreases in plasma lipids via dietary modification on the extent of atherosclerosis. The plasma lipoprotein and coronary artery atherosclerosis portions of this study have been published separately.[29] In brief, dietary lipid decreases allowed remodeling of the coronary arteries, and addition of hormone replacement did not further augment this response. We report here the effects of

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Submitted January 4, 1996; accepted April 24, 1996.

Supported in part by Grants No. KO1 RR00072 (J.D.W.) and No. PO2-RR08562 (J.D.W. and W.T.C.) from the National Center for Research Resources, Grants No. HL-38964 and PO1-HL-45666 from the National Heart, Lung, and Blood Institute, and Grant No. KO1-AG000578 (W.T.C.) from the National Institute on Aging, National Institutes of Health, Bethesda, MD.

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0026-0495/96/4510-0011$03.00/0

hormone replacement therapy on carbohydrate metabolism and body composition in surgically postmenopausal cynomolgus monkeys and relate these parameters to atherosclerosis.

MATERIALS AND METHODS

Animal Studies

This study used 68 adult female cynomolgus monkeys that were imported from Indonesia (Charles River Primates, Port Washington, NY) as part of a separate study to determine the effects of dietary lipid decreases on atherosclerosis.[29] The age of the monkeys was estimated by dentition, and ranged from 5 to 13 years and averaged 13.8 ± 0.5, 14.1 ± 0.5, and 13.7 ± 0.2 years for the three groups (described below) at the end of the study ($P > .05$). After quarantine, the monkeys were ovariectomized and fed a moderately atherogenic diet containing 43% of calories from fat and 0.44 mg cholesterol/kcal for 2 years, resulting in total plasma cholesterol concentrations of approximately 17 mmol/L.[29] After 2 years of the atherosclerosis progression phase, the monkeys were fed a plasma lipid-lowering diet containing 31% of calories as fat and 0.05 mg cholesterol/kcal for an additional 30 months. Following the progression phase, the monkeys were randomized into one of three treatment groups based on (1) total plasma cholesterol to high-density lipoprotein cholesterol ratio, (2) most recent bone density measurement (8 months before beginning treatment), and (3) time since ovariectomy.[29] One group (n = 25) was fed the plasma lipid-lowering diet alone (controls). The second group (n = 22) was fed the plasma lipid-lowering diet plus conjugated equine estrogens ([CEE] Premarin; Wyeth-Ayerst, Radnor, PA) at a dose of 166 μg/d, which is equivalent to a woman receiving 0.625 mg/d. The third group (n = 21) was fed the plasma lipid-lowering diet plus CEE at doses as above and medroxyprogesterone acetate ([MPA] Cycrin; Wyeth-Ayerst) at a dose of 650 μg/d, which is equivalent to a woman receiving 2.5 mg/d. The hormones were administered twice per day in the diet.

At the time of necropsy, the monkeys were sedated with ketamine hydrochloride (15 mg/kg intramuscularly) and then anesthetized to a surgical plane of anesthesia with sodium pentobarbital 80 mg/kg intravenously (a method consistent with recommendations of the American Veterinary Medical Association panel on euthanasia). All procedures involving animals were conducted in compliance with state and federal laws, standards of the US Department of Health and Human Services, and guidelines established by the Institutional Animal Care and Use Committee.

Clinical Chemistry Measurements

Plasma lipid and lipoprotein concentrations were measured quarterly throughout the study and have been reported separately.[29] To assess the adequacy of dietary hormone delivery, one estrogen component of CEE (17β-estradiol) and MPA levels were determined in serum 4 hours after the diets were given (ie, at peak concentrations) as described previously.[29] In brief, estradiol concentrations were measured using a modification of a commercially available kit (Diagnostic Products, Los Angeles, CA). MPA concentrations were measured by radioimmunoassay after samples were extracted with diethyl ether, yielding a recovery of 91.2%. The antibody was a rabbit anti-MPA (American Biochemicals, San Diego, CA). In addition, serum androstenedione, dehydroepiandrosterone sulfate (DHEAS), testosterone (all measured 4 hours postfeeding), and fasting insulin-like growth factor-1 (IGF-1) concentrations were measured at the Comparative Endocrinology Laboratory at Yerkes Regional Primate Center of Emory University (Atlanta, GA). Androstenedione and testosterone levels were measured at months 20 and 25 of treatment and DHEAS levels at month 25 using commercial radioimmunoassay kits from Diagnostic Products. Fasting IGF-1 measurements (month 25) were made using reagents from the National Hormone and Pituitary Program Awards Management System.

Fasting glucose and insulin concentrations and intravenous glucose tolerance tests (IVGTTs) with insulin responses were determined as described previously[28] during the 13th month of the progression phase (pretreatment) and at the 13th and 23rd month of the treatment phase. The monkeys were sedated with ketamine hydrochloride (15 mg/kg intramuscularly), and IVGTTs were performed by injecting a 50% dextrose solution at 750 mg/kg into each monkey. After taking a baseline sample, subsequent blood samples were taken at 5, 10, 20, 30, 40, and 60 minutes. The rate of glucose disappearance (k value) was calculated from the 5-, 10-, and 20-minute samples. In addition to basal glucose and insulin, the areas under the curve (AUCs) for glucose and insulin were calculated by using all of the time points of the IVGTT. Glucose determinations were made using glucose oxidase methodologic techniques on a Beckman Analyzer 700 (Fullerton, CA). Insulin concentrations were determined by radioimmunoassay procedures (Diagnostic Products) at the Yerkes Regional Primate Center Comparative Endocrinology Laboratory.[28]

Body Measurements and Composition

Abdominal body composition was determined by scans at the third lumbar vertebra (L3) using dual-energy x-ray absorptiometry ([DEXA] XR26; Norland, Fort Atkinson, WI)[30] at 8 months pretreatment and after 17, 22, and 26 months of treatment. A subset of animals that underwent necropsy at baseline (n = 20) were used to determine the precision of the DEXA spine methodology. Briefly, two DEXA scans were taken without repositioning at 1.5-mm line spacing, 1.5-mm point resolution, a speed of 60 mm/s, and a width of 6 cm. Analyses of the DEXA scan provided the soft, lean, and fat content of this segment. The total time per scan was less than 5 minutes. The reproducibility for L3 soft, L3 lean, and L3 fat tissue determinations by in vivo DEXA was excellent as determined by the correlation coefficient between the two scans for each measurement ($r = .993, .987$, and .994, respectively, all $P < .001$). Body weight and body mass index were measured at the same time and were correlated significantly ($P < .05$) with L3 soft and L3 fat but not with L3 lean tissue.

Body weight was measured a number of times during the study. Waist circumference was taken at the umbilicus, which corresponds to the area of the third lumbar vertebra, after 24 months of treatment.

Fat Cell Size Measurements

Three 1- to 4-g samples of adipose tissue were collected at necropsy: one from the femoral region, one from the omental fat, and one from the subcutaneous abdominal fat. The tissues were placed immediately in 0.9% saline on ice and processed within 30 minutes of collection. Tissues were digested at 37°C in Parker media (medium 199 containing 4% albumin [wt/vol]; Life Technologies, Grand Island, NY) with 1.5 mg/mL collagenase (Sigma, St Louis, MO) for 60 minutes. The samples were then filtered through a 250-μm nylon mesh and washed three times with Parker media. A 100-μL sample was stained with a drop of 0.25% crystal violet solution. Stained cells were placed on siliconized glass slides, and fat cell size was determined by measuring the diameter of 100 cells with a Summagraphics morphometer and software (Woods Hole Educational Associates, Woods Hole, MA). The mean ± SEM were then determined.

Arterial and Skin Glycation Measurements

AGE were assessed in skin and aorta by fluorescence. An elliptical full-thickness skin section (0.5 × 1 cm) from the abdomen and a 1-cm ring from the aorta (just above the celiac bifurcation) were removed at necropsy, rinsed in saline, and stored at −70°C until assayed. As described previously,[31] tissues were thawed and scraped vigorously with a scalpel to remove adherent fat, and then extracted sequentially for 24 hours at 4°C with 1 mol/L NaCl, chloroform:methanol (2:1), and 0.5N acetic acid to remove lipids and soluble proteins. After extraction, the samples were rinsed in distilled water, dried by lyophilization, and stored at −70°C. Approximately 20 to 30 mg protein was obtained from each sample. Tissue glycation was determined as total fluorescence after collagen digestion with 1% pepsin (wt/wt) in 0.25 mol/L acetic acid for 24 hours. Fluorescence was measured at excitation and emission wavelengths of 325 and 375 nm, respectively, and reported as total fluorescence (units per milligram dry weight) and then normalized for hydroxyproline content (units per milligram hydroxyproline).

Atherosclerosis Evaluation

Coronary and aortic atherosclerosis were determined as described previously.[29] Aortic atherosclerosis was determined as the mean of three sections below the celiac bifurcation. Coronary artery atherosclerosis was determined as the mean of the three main coronary arteries (the right, left circumflex, and left anterior descending coronary arteries). Atherosclerosis extent is reported as the cross-sectional area inside the internal elastic lamina divided by the length of the internal elastic lamina (mm^2/mm) to correct for differences in artery size.

Statistical Analyses

Reported values are the mean ± SEM. All analyses were made using BMDP Statistical Software (Version 7.0; Los Angeles, CA). Analyses were made on log-transformed data if Levene's test for homogeneity of variances was significant ($P < .05$), indicating inequality of variances. Repeated-measures ANOVA or ANCOVA were used for all variables for which there were repeated measures. If there was a significant treatment group effect, pairwise comparisons between treatment groups were made by adjusting for multiple comparisons with the Bonferroni method. Pearson product-moment correlations were used to assess the relationship among variables. Statistical significance was set at 95%.

RESULTS

Clinical Chemistry Measurements

Longitudinal changes in fasting glucose, insulin, and the insulin to glucose ratio are shown in Fig 1. No significant differences were found before treatment, but after 13 and 23 months of treatment both glucose and insulin concentrations and the insulin to glucose ratio were significantly increased in the combined CEE + MPA group compared with controls and CEE-treated monkeys.

Changes in plasma glucose and insulin concentrations in response to the glucose challenge are shown in Fig 2, both before treatment and after 23 months of treatment. Although there were no significant differences before treatment, the insulin responses to the glucose challenge (Fig 2C) were highest in the control group and lowest in the CEE + MPA group. However, with treatment, the CEE + MPA group had increased basal insulin levels and insulin responses up to 30 minutes (Fig 2D), resulting in a greater AUC (see below). No significant differences were found in

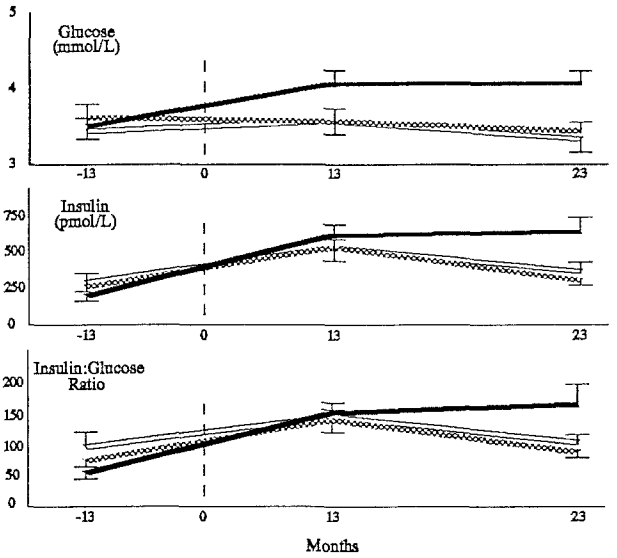

Fig 1 Changes in fasting glucose and insulin concentrations with time for ovariectomized control monkeys (☐) and those treated with CEE (----) and CEE + MPA (—). Effect of treatment on glucose concentrations as determined by ANCOVA, $P = .002$; effect of time, $P > .10$; treatment × time interaction, $P > .10$. Effect of treatment on insulin concentrations as determined by ANCOVA, $P = .009$; effect of time, $P = .04$; treatment × time interaction, $P > .10$. Effect of treatment on insulin to glucose ratio as determined by ANCOVA, $P = .02$; effect of time, $P = .07$; treatment × time interaction, $P > .10$.

the plasma glucose response to the glucose challenge during treatment (Fig 2B).

Adjusted means for the glucose and insulin measurements determined from the IVGTT are summarized in Table 1. In addition to increases in glucose and insulin concentrations with CEE + MPA treatment, there were increases in the insulin to glucose ratio (values obtained as zero-time points of IVGTT), the AUC for insulin, and the ratio of the AUC for insulin to the AUC for glucose in the CEE + MPA group compared with both the control and CEE groups. There were no significant differences in the AUC for glucose or the rate of glucose disappearance with treatment.

In addition to treatment effects, except for fasting glucose concentrations, there were significant changes with time as indicated by a significant repeat effect (all $P < .05$). The changes with time were due to decreases in fasting insulin levels and glucose and insulin AUCs, as well as decreases in insulin to glucose ratios, from 13 to 23 months. There was also a decrease in glucose disappearance with time. Although the CEE + MPA group did not appear to change as much during the treatment period, there was no significant time-by-group interaction (all $P \geq .1$), suggesting similar changes with time for all groups.

There were no significant differences in mean total plasma cholesterol concentrations among the groups during the treatment phase (control, 4.21 ± 0.15 mmol/L; CEE, 3.96 ± 0.15 mmol/L; and CEE + MPA, 4.14 ± 0.18 mmol/L). However, triglyceride concentrations were significantly increased in the CEE group (1.30 ± 0.07 mmol/L) compared with the control and CEE + MPA groups (0.75 ± 0.07 and 0.96 ± 0.07 mmol/L, respectively, $P = .001$). More

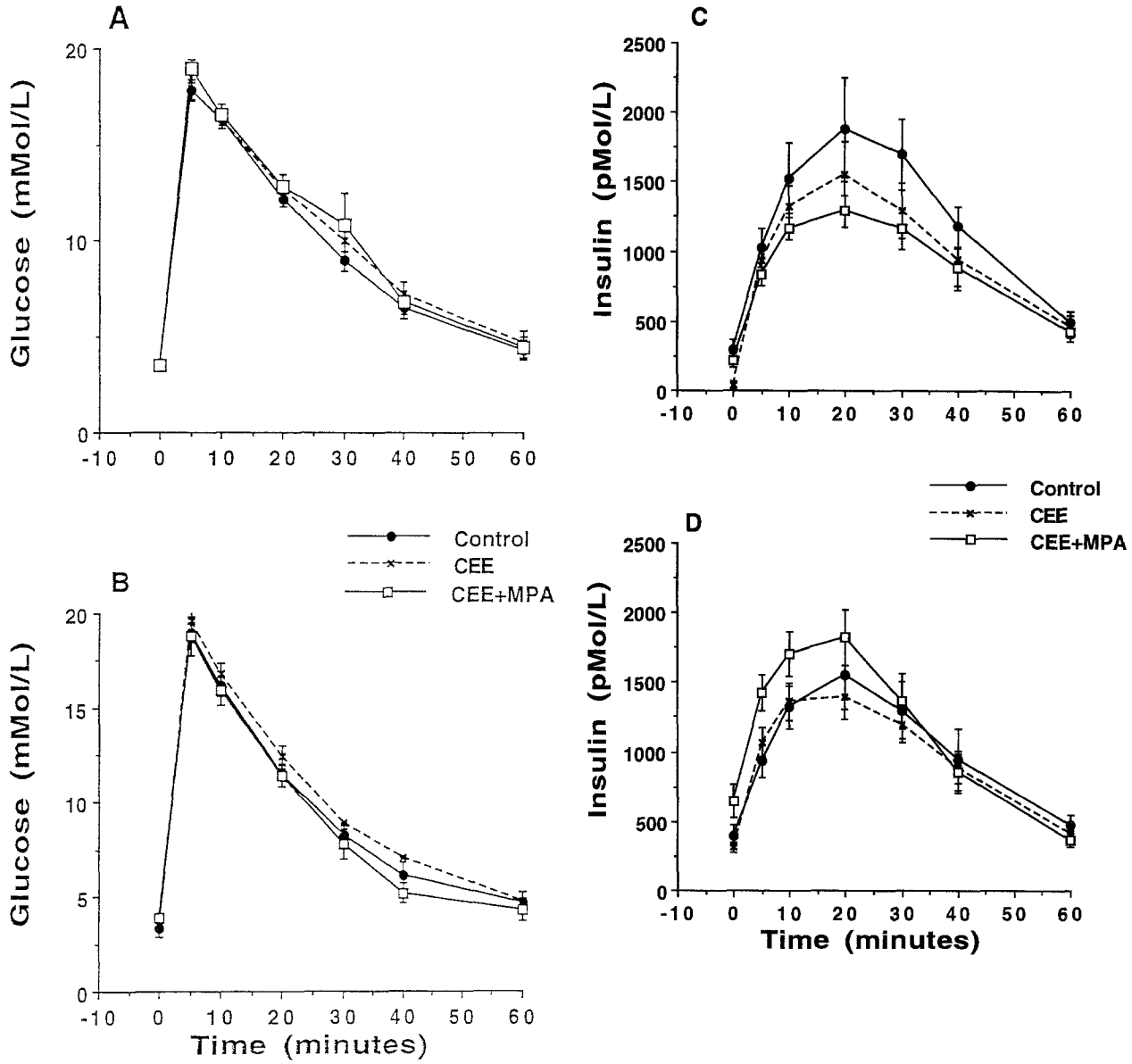

Fig 2. Changes in glucose and insulin concentrations in response to an IVGTT before treatment (A and C) and after 23 months of treatment (B and D) for ovariectomized control monkeys and those treated with CEE and CEE + MPA.

detailed lipid and lipoprotein determinations have been reported separately.[29]

As reported previously,[29] ovariectomized control monkeys had very low estradiol and MPA concentrations (5.0 and 25 pg/mL, respectively). There were no differences in serum estradiol between CEE and CEE + MPA groups (167 ± 10 and 161 ± 14 pg/mL, respectively), whereas MPA concentrations were elevated only in CEE + MPA

Table 1. Effect of Hormone Treatment on Plasma Glucose and Insulin Levels (adjusted mean ± SEM)

Parameter	Control	CEE	CEE + MPA	P*	Pairwise Comparisons†
Glucose (mmol/L)	3.47 ± 0.13	3.42 ± 0.14	4.07 ± 0.14	.002	Control ≠ CEE + MPA, CEE ≠ CEE + MPA
Insulin (pmol/L)	457 ± 56	402 ± 59	662 ± 61	.009	Control ≠ CEE + MPA, CEE ≠ CEE + MPA
Insulin to glucose ratio	124 ± 13	111 ± 14	166 ± 15	.02	CEE ≠ CEE + MPA
Glucose disappearance AUC	534 ± 23	580 ± 24	538 ± 25	.32	—
Insulin disappearance AUC	64,891 ± 10,505	61,153 ± 11,088	103,715 ± 11,563	.02	Control ≠ CEE + MPA, CEE ≠ CEE + MPA
Disappearance AUC, insulin to glucose ratio	130 ± 13	115 ± 14	187 ± 14	.0015	Control ≠ CEE + MPA, CEE ≠ CEE + MPA
Rate of glucose disappearance (k value)	3.90 ± 0.37	3.27 ± 0.41	3.84 ± 0.43	.24	—

*Analyses by repeated-measures ANOVA with baseline values as covariates (ANCOVA).

†Pairwise comparisons between group means adjusted for baseline value. Significant differences between groups ($P < .05$) after Bonferroni adjustment for multiple comparisons.

Table 2. Effect of Hormone Treatment on Plasma Hormone Concentrations (mean ± SEM)

Hormone	Control	CEE	CEE + MPA	*P* (ANOVA)	Pairwise Comparisons*
Androstenedione (nmol/L)	6.7 ± 0.7	4.2 ± 0.4	5.8 ± 0.9	.05	Control ≠ CEE
DHEAS (nmol/L)	0.22 ± 0.03	0.09 ± 0.03	0.15 ± 0.03	.01	Control ≠ CEE
Testosterone (nmol/L)	0.60 ± 0.06	0.51 ± 0.08	0.70 ± 0.14	.40	—
IGF-1 (ng/mL)†	152	137	221	.007	Control ≠ CEE + MPA, CEE ≠ CEE + MPA

*Pairwise comparisons between group means. Significant differences between groups ($P < .05$) after Bonferroni adjustment for multiple comparisons.

†Means are in the original units derived by retransforming the means of the transformed data. Standard errors are not included, since they are not equal excursions from the retransformed mean. Comparisons made on log-transformed data.

monkeys (116 ± 5 pg/mL). Additional hormone determinations are shown in Table 2. Androstenedione and DHEAS were both significantly lower in CEE-treated animals compared with controls, with a similar trend for testosterone. IGF-1 concentrations were significantly increased in the CEE + MPA group compared with both control and CEE-treated monkeys. DHEAS concentrations correlated inversely with age in control monkeys ($r = -.60$, $P < .01$), but hormone treatments negated this correlation.

Body Composition and Fat Cell Size

Changes in body weight and abdominal body composition with time are shown in Fig 3. No significant difference in any measurement was found before treatment. However, with time, there was an increase in body weight in the control and CEE + MPA groups compared with the CEE monkeys ($P = .04$). The increase in body weight was mirrored by an increase in abdominal soft tissue mass as determined by DEXA ($P < .05$). The change in soft tissue mass was due to changes in fat tissue with no significant change in lean tissue during the treatment phase. Summarized in Table 3 are the adjusted means for these measurements.

There were also changes in body weight and soft and fat tissue with time (all $P < .001$; Fig 3). In addition, there were time-by-treatment interactions for these measures (all $P \leq .03$). These differences were due to relatively small changes during treatment in the CEE group compared with

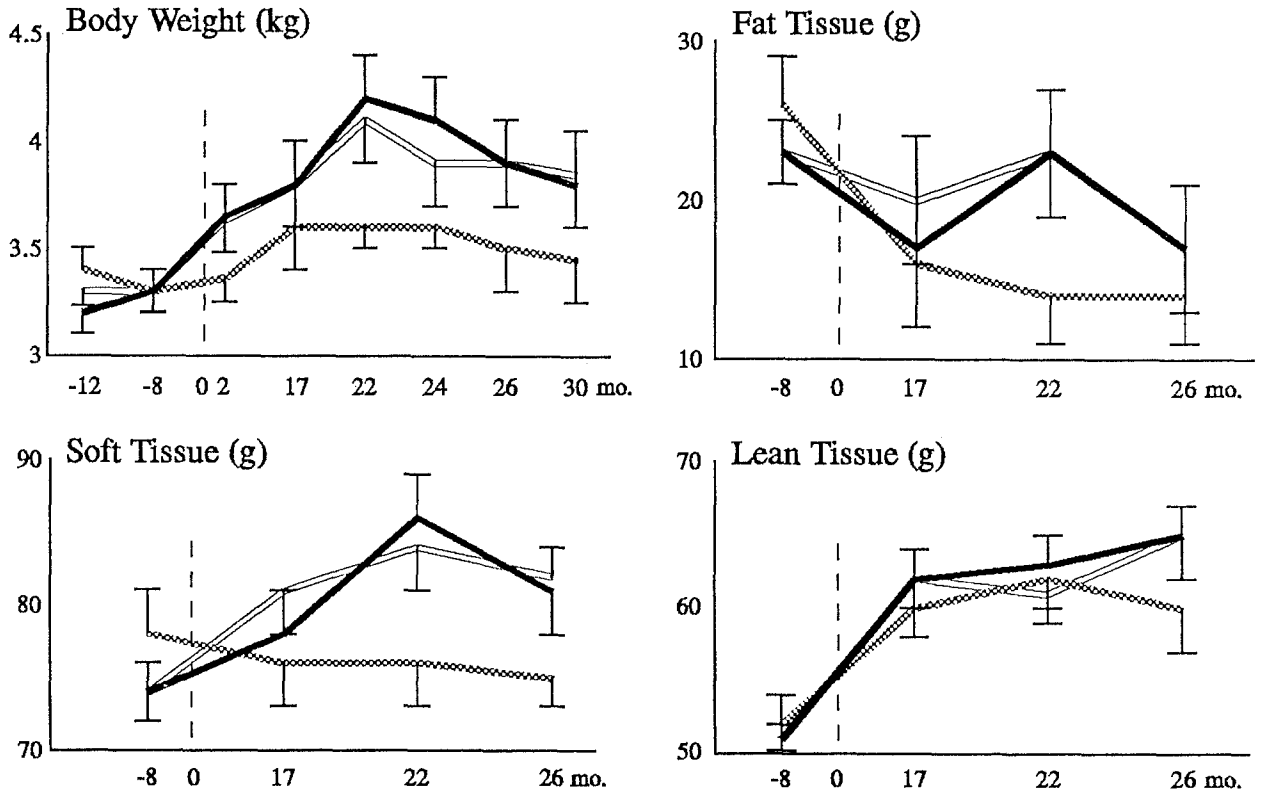

Fig 3. Changes in body weight and abdominal body composition over time for ovariectomized control monkeys (▭) and those treated with CEE (----) and CEE + MPA (—). Effect of treatment on body weight as determined by ANCOVA, $P = .04$; effect of time, $P = .0001$; treatment × time interaction, $P = .0005$. Effect of treatment on soft tissue as determined by ANCOVA, $P = .007$; effect of time, $P = .003$; treatment × time interaction, $P = .02$. Effect of treatment on fat tissue as determined by ANCOVA, $P = .056$; effect of time, $P = .0003$; treatment × time interaction, $P = .03$. Effect of treatment on lean tissue as determined by ANCOVA, $P = .33$; effect of time, $P = .36$; treatment × time interaction, $P = .26$.

Table 3. Effect of Hormone Treatment on Body Weight and Abdominal Body Composition (adjusted mean ± SEM)

Site	Control	CEE	CEE + MPA	P* ANCOVA	P* Time × Effect	P* Time × Group	Pairwise Comparisons†
Soft tissue (g)	82.9 ± 3.5	74.5 ± 3.7	83.3 ± 3.7	.007	.0003	.015	Control ≠ CEE + MPA, CEE ≠ CEE + MPA
Fat tissue (g)	20.4 ± 3.7	13.3 ± 3.9	19.6 ± 3.9	.056	.0003	.03	—
Lean tissue (g)	62.6 ± 2.4	60.8 ± 2.5	63.9 ± 2.5	.33	NS	NS	—
Body weight (kg)	3.92 ± 0.20	3.59 ± 0.21	4.03 ± 0.22	.04	.0001	.0005	CEE ≠ CEE + MPA

*Analyses by repeated-measures ANOVA with baseline values as covariates (ANCOVA).

†Pairwise comparisons between group means. Significant differences between groups ($P < .05$) after Bonferroni adjustment for multiple comparisons.

increases in the control and CEE + MPA groups. There was no change in lean tissue during the treatment phase in any group.

Consistent with lower body weight and central soft/fat tissue with CEE treatment, waist circumferences were significantly smaller ($P = .01$) in CEE monkeys (22.6 ± 0.6 cm) compared with control and CEE + MPA groups (26.2 ± 1.1 and 27.4 ± 1.6 cm, respectively). Waist circumference was significantly correlated ($P < .001$) with both the mean abdominal fat and soft tissue measured by DEXA ($r = .86$ and .78, respectively) and mean body weight ($r = .87$).

Fat cell sizes from the three different sites (femoral, omental, and subcutaneous abdominal) are shown in Table 4. Subcutaneous abdominal adipocytes were significantly larger than femoral adipocytes ($P = .03$) and slightly larger than omental adipocytes ($P = .2$) across all treatment groups. Although fat cell sizes were not significantly different among treatment groups, both hormone treatments tended to decrease adipocyte size ($P = .22$, repeated-measures ANOVA). This difference was greatest in omental fat ($P = .14$). Omental fat cell size correlated significantly ($P < .001$) with waist circumference ($r = .58$) and abdominal fat tissue ($r = .69$), as did femoral and subcutaneous adipocytes (data not shown).

Glycation and Atherosclerosis Measurements

Aortic and skin glycation determinations are shown in Table 5. AGE, as determined by total fluorescence, were significantly increased in CEE monkeys compared with both control and CEE + MPA monkeys. When the fluorescence was corrected for the amount of hydroxyproline present, there was still a significant increase in skin glycation, with a similar trend in the aorta.

There was no significant correlation between fasting glucose levels and arterial glycation, but there was a relationship between the glucose AUC and total and normalized arterial glycation ($r = .22$ and .21, respectively, $P < .10$). Furthermore, when all monkeys were stratified into two groups based on plasma glucose AUC during treatment, animals with lower AUCs for plasma glucose disappearance had significantly less arterial glycation compared with those with higher AUCs (70 ± 22 *v* 83 ± 26 U/mg hydroxyproline, respectively, $P < .05$). In addition, there was a significant correlation between animal age and total arterial glycation within each treatment group, as well as for all animals together ($r = .43$, .51, and .46 for control, CEE, and CEE + MPA groups, respectively, all $P < .05$; $r = .47$ for all animals, $P < .001$).

There was no significant effect of hormone treatment in addition to the lipid-lowering diet on coronary artery or aortic atherosclerosis (Table 5). There was also no significant correlation between measures of aortic glycation and aortic or coronary artery atherosclerosis ($P > .10$).

Table 4. Effect of Hormone Treatment on Adipocyte Size (μm, mean ± SEM)

Site	Control	CEE	CEE + MPA	ANOVA*
Femoral	98.36 ± 4.64	90.94 ± 3.18	93.17 ± 4.34	.50
Intraabdominal	107.12 ± 6.07	90.51 ± 6.0	92.12 ± 6.0	.14
Subcutaneous abdominal	110.18 ± 5.41	98.07 ± 6.3	100.13 ± 5.8	.29

*Analyses by repeated-measures ANOVA.

Correlational Analyses

Shown in Table 6 are correlations between mean fasting insulin concentrations and other measures (during the treatment phase). Insulin levels were significantly associated with glucose concentrations, androgen levels, and measures of central adiposity. These correlations were greatest within the control group. There was no correlation between insulin levels and coronary artery atherosclerosis.

DISCUSSION

Monkeys receiving combined estrogen and progestin therapy had significantly higher plasma glucose and insulin concentrations compared with control monkeys and those receiving estrogen alone. In addition, combined hormone therapy was associated with a higher fasting insulin to glucose ratio and a higher ratio for the insulin AUC to glucose AUC, further suggesting insulin resistance. These results agree with our previous report of hormone therapy after only 12 weeks of treatment in the same animal model.[18] In our previous study,[18] we determined insulin sensitivity and glucose effectiveness by minimal-model analyses and found no change in glucose effectiveness, whereas insulin sensitivity was decreased by approximately 50% in MPA-alone or CEE + MPA groups compared with controls and CEE monkeys. Thus, both short- and long-term combination hormone treatment was associated with insulin resistance.

Table 5. Effect of Hormone Treatment on Tissue Glycation and Atherosclerosis (mean ± SEM)

Parameter	Control	CEE	CEE + MPA	ANOVA	Pairwise Comparisons*
Aortic glycation					
Total fluorescence (U/mg dry weight)	1.30 ± 0.06	1.53 ± 0.007	1.24 ± 0.04	.003	Control ≠ CEE, CEE ≠ CEE + MPA
Normalized (U/mg hydroxyproline)	71 ± 4	85 ± 6	75 ± 6	.15	—
Skin glycation					
Total fluorescence (U/mg dry weight)	0.15 ± 0.01	0.19 ± 0.01	0.15 ± 0.01	.003	Control ≠ CEE, CEE ≠ CEE + MPA
Normalized (U/mg hydroxyproline)	4.9 ± 0.2	5.9 ± 0.3	4.8 ± 0.2	.001	Control ≠ CEE, CEE ≠ CEE + MPA
Aortic atherosclerosis (mm^2/mm)	0.204 ± 0.021	0.183 ± 0.022	0.157 ± 0.016	.25	—
Coronary artery atherosclerosis (mm^2/mm)	0.075 ± 0.009	0.064 ± 0.009	0.073 ± 0.011	.68	—

*Pairwise comparisons between group means. Significant differences between groups ($P < .05$) after Bonferroni adjustment for multiple comparisons.

Previous studies of the effects of hormone replacement therapy on carbohydrate and insulin metabolism have yielded conflicting results. This is in part due to various methods of assessing insulin sensitivity/resistance, as well as different types of estrogens and progestins studied and different routes (oral *v* transdermal) and methods of administration (continual or sequential progestin coadministration). However, a few large epidemiologic studies, including the Atherosclerosis Risk in Communities Study,[32] the Rancho Bernardo Study,[33] and the Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial,[34] have reported small but significant improvements in glucose and/or insulin levels using primarily CEE with or without MPA therapy. Unfortunately, in those studies, the investigators do not describe when sampling was performed with respect to varying progestin treatment schedules in women treated with sequential progestin coadministration. This is an important additional piece of information since, as reported by Godsland et al,[15] there is an increase in insulin resistance during the progestin phase of sequential combined hormone therapy compared with estrogen alone. Furthermore, insulin sensitivity has even been shown to decrease during the luteal phase of the menstrual cycle when progesterone levels are higher.[35] Thus, as reported with progestin use in oral contraceptive therapy,[36,37] progestin use in combined hormone replacement therapy may also have adverse effects on insulin resistance. In the present study and our previous report,[18] an oral progestin (MPA) was given continuously with CEE, which would theoretically increase the chances of detecting insulin resistance compared with either sequential or perhaps transdermal progestin use.

Table 6. Correlations Between Mean Plasma Insulin Concentrations and Other Parameters

Parameter	All (N = 68)	Control (N = 25)	CEE (N = 22)	CEE + MPA (N = 21)
Glucose	.60*	.69*	.57†	.34
Androstenedione	.26‡	.54†	.36	−.03
DHEAS	.23	.46‡	.36	−.04
Testosterone	.13	.44‡	.02	−.05
Body weight	.44*	.61†	.31	.26
Waist circumference	.41*	.41‡	.10	.32
Abdominal fat	.50*	.53†	.62†	.44†
Coronary artery atherosclerosis	.10	−.05	.16	.15

*$P < .001$.
†$P < .01$.
‡$P < .05$.

Whereas some studies of estrogen therapy alone have reported a decrease of glucose and insulin[32-34] or an improvement in insulin sensitivity,[12,13,17] others have not.[15,16] In the present study, CEE alone did not significantly decrease fasting glucose or insulin concentrations compared with levels in controls. However, there was a trend toward an improvement in insulin resistance, as suggested by the decreased fasting insulin levels and insulin to glucose ratios (both fasting and AUC values). Perhaps with more subjects or the use of more sensitive techniques (ie, minimal-model studies), a significant beneficial effect might have been found. Interestingly, despite having the lowest fasting glucose level, the CEE group had a slightly higher glucose AUC (due to higher glucose levels at later time points in the IVGTT; Fig 2B) than either the control or CEE + MPA groups. This is consistent with the results of the PEPI Trial,[34] which found an increase in the 2-hour glucose levels (following an oral GTT) compared with levels in the placebo group. Godsland et al[15] also reported a deterioration in glucose tolerance, and suggested that it was due to the decreased plasma insulin levels with estrogen.

Although it would seem that the small and not statistically significant increase in the glucose AUC would not be biologically important, it is interesting that CEE-treated animals had the greatest amount of tissue glycation (in both the aorta and skin). In addition, when the animals were stratified into two groups based on glucose AUC, those with higher glucose levels had greater arterial glycation. Protein glycation is dependent on both the half-life of the protein in the circulation or tissue and the degree and duration of hyperglycemia.[22,23] As such, the extent of glycation of numerous proteins is increased in proportion to the mean blood glucose concentration, particularly in diabetes.[23] Also, advanced glycation of collagen, as measured by fluorescence, obtained from the dura mater of normal subjects was found to increase linearly with age.[38] Thus, the correlation between glucose AUC and age in the present study is consistent with previous reports in humans.

Insulin resistance has been found to be an independent risk factor for atherosclerosis after correcting for age, ethnicity, gender, body composition, and glucose and insulin levels.[5] However, the determinants of in vivo insulin resistance are significantly related to body composition.[39] In

this study, monkeys receiving CEE treatment had significantly lower body weights and waist circumferences than either the control or CEE + MPA groups. In addition, CEE-treated monkeys had less abdominal body fat and smaller adipocytes. Furthermore, all measures of adiposity correlated with fasting insulin levels. CEE-treated monkeys also had lower androgen levels, which previously have been associated with central adiposity. In women, hormone replacement therapy prevents the increase in abdominal fat that occurs after menopause,[20] and as shown in both the ARIC and PEPI studies, women treated with hormone replacement therapy weigh less than those who are not treated,[32,34] with those receiving CEE alone weighing the least.[34] Also, combined hormone treatment significantly decreased abdominal fat as determined by DEXA[20] and by impedance.[40] Thus, the beneficial effect of estrogens on CHD risk may partly be explained by the prevention of abdominal fat deposition.

The decreased central adiposity and lower androgen levels may explain the improved insulin resistance in CEE-treated monkeys compared with those receiving combined hormone therapy. However, it is interesting that the control monkeys had body weight and abdominal ponderosity similar to those of CEE + MPA monkeys yet had glucose and insulin levels similar to those of CEE monkeys. A limitation of the methodology reported herein is that DEXA does not distinguish between subcutaneous and intraabdominal fat. This is important, since intraabdominal fat has been reported to be metabolically most active,[41] and we have found in both men and women[42] and in monkeys[43] that it predicts insulin sensitivity better than total abdominal fat. However, in addition to changes in fat, there are potential changes in lean tissue. Muscle in particular is most responsible for peripheral insulin resistance and may also be affected by hormone therapies. These areas are currently under investigation.

There have been conflicting reports in the literature as to whether a combination of estrogen and progestin has beneficial or deleterious effects on carbohydrate metabolism. Our results suggest that continual MPA administration in combination with CEE treatment results in insulin resistance. Since dietary lipid-lowering therapy was so effective in this study that hormone therapies had no additional effect on atherosclerosis extent, we were limited in our ability to determine how changes in insulin resistance, arterial glycation, and body composition affected atherosclerosis extent. However, in a separate investigation using the same hormone replacement therapies but studying atherosclerosis progression, we found a beneficial effect of CEE treatment on coronary artery atherosclerosis that was diminished by the addition of MPA,[44] also suggesting adverse effects of MPA. Future studies are under way to determine the effect of insulin resistance, hyperglycemia, and arterial AGE on the progression of atherosclerosis.

ACKNOWLEDGMENT

The authors thank Marielle Rebuffé-Scrive, PhD, for assistance in determining fat cell size, and we also acknowledge the technical assistance of Vickie Hardy, Maryanne Post, Sam E. Rankin, Audrey Bell-Farrow, and Zhong Q. Wang, MD, and the editorial assistance of Karen Potvin Klein.

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