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17β-Estradiol Supplementation Attenuates Ovariectomy-Induced Increases in ATGL Signaling and Reduced Perilipin Expression in Visceral Adipose Tissue

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ABSTRACT

Adipocytes from post-menopausal females have higher basal lipolytic rates than pre-menopausal females, which contributes to increased risk of developing dyslipidemia following menopause. The purpose of this study was to delineate cellular mechanisms affecting adipose tissue function in the ovariectomized (OVX) mouse and also determine if physical activity or estrogen supplementation alter any detected changes. Female C57/Bl6 mice were placed into SHAM, OVX sedentary (OVX), OVX exercise (OVX-Ex), and OVX sedentary + 17β-estradiol (OVX + E_2) groups. Visceral fat mass, glycerol, and NEFA levels were significantly higher in OVX mice compared to SHAM animals, but were not elevated in the E_2-treated animals. Voluntary running failed to change circulating levels of glycerol or NEFA in OVX mice, but did partially attenuate the increase in visceral fat mass. Adipose triglyceride lipase (ATGL) protein content was significantly elevated in visceral fat from OVX and OVX-Ex groups compared to SHAM, while ATGL–CGI-58 interaction was significantly higher in OVX than SHAM and OVX + E_2 mice. No significant differences in HSL phosphorylation were detected between groups, however, ERK1/2 phosphorylation was significantly elevated in the OVX mice. To determine if ERK1/2 function was critical for the increased glycerol levels, visceral fat was treated with MEK inhibitor PD98059, with no differences in glycerol release detected. Perilipin protein content was decreased significantly in OVX and OVX-Ex mice compared to SHAM. Thus, these data suggest that increased ATGL signaling and reduced perilipin protein content may contribute to increased NEFA and glycerol levels in OVX mice, which are attenuated with E_2 treatment, but not by exercise. J. Cell. Biochem. 110: 420–427, 2010.

KEY WORDS: MENOPAUSE; LIPID; HORMONE SENSITIVE LIPASE; ADIPOCYTE

It is well documented that as women transition into menopause, those who do not take hormone replacement therapy (HRT) demonstrate a disproportionate increase in lipid storage with an apparent decline in lipid utilization [Ferrara et al., 2002]. Loss of ovarian function also leads to an increase in visceral fat mass, which concurrently results in an increased lipolytic rate [Panotopoulos et al., 1997; Schneider et al., 2006; Dubnov-Raz et al., 2007]. The link between ovarian hormones and alterations in metabolism has been demonstrated in both human and animal models [Laudenslager et al., 1980; Panotopoulos et al., 1997; Shinoda et al., 2002; D'Eon et al., 2005]. The enhanced lipolytic rate is particularly problematic because it may contribute to the development of peripheral insulin resistance and ectopic lipid deposition in the liver, both of which have been observed in animals with reduced ovarian function [Paquette et al., 2009; Saengsirisuwan et al., 2009]. These conditions result in an increased risk for developing cardiovascular disease (CVD) and other lipid-based conditions [Carr, 2003; Rosano et al., 2006]. In addition to ectopic lipid deposition in liver tissue, a high level of circulating lipid places the individual at increased risk for ectopic fat deposition in skeletal muscle [Goodpaster and Brown, 2005]. Furthermore, post-menopausal women are at increased risk for developing insulin resistance. Therefore, it is critical to delineate the mechanisms which are affecting metabolic function in post-menopausal women.

Triacylglycerols (TAG) are stored and utilized as energy substrates primarily by adipose tissue and skeletal muscle, respectively. To oxidize free fatty acids (FFA), the FFA must be liberated from the TAG through enzymatic-regulated hydrolysis of the stored TAG. Although hormone sensitive lipase (HSL) was long believed to be the rate-limiting lipase, several recent studies have demonstrated the importance of adipose triglyceride lipase (ATGL) to lipolysis [Ferrara et al., 2002; Watt and Steinberg, 2008; Watt, 2009]. ATGL, also known as desnutrin, was found to catalyze the first step in lipid hydrolysis, breaking down TAG to diacyglycerol (DAG) and

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releasing a FFA [Villena et al., 2004; Zimmermann et al., 2004]. HSL subsequently cleaves another FFA, converting DAG to monoacylglycerol (MAG). The final step of lipid hydrolysis is catalyzed by monoacylglyceride lipase (MGL), which breaks down MAG to glycerol and a FFA, which are then released into systemic circulation [Fredrikson et al., 1986]. Thus, elucidating the mechanisms regulating ATGL and HSL activity is a critical step toward understanding lipid metabolism.

The complete activation of ATGL is thought to be regulated by protein–protein interaction and/or by phosphorylation, albeit very little is known about regulation of ATGL through phosphorylation [Brasaemle, 2007]. A number of potential phosphorylation sites have been suggested for ATGL, but at this time none have been documented to be necessary for complete lipase activity [Zimmermann et al., 2004]. For example, ATGL contains an ERK1/2-specific phosphorylation motif, suggesting that ATGL may be sensitive to changes in MAPK activation, although this has never been measured [Bartz et al., 2007]. However, it is well known that complete ATGL activation is dependent upon interaction with comparative gene identification-58 (CGI-58) [Lass et al., 2006]. CGI-58, also known as abhydrolase domain containing 5, plays a critical role in the autosomal recessive disease Chanarin–Dorfman syndrome, which is characterized by excessive TAG accumulation in various tissues [Duncan et al., 2007].

Unlike ATGL, the cellular regulation of HSL is well understood. HSL is phosphorylated at five different serine residues (Ser563, Ser565, Ser600, Ser659, and Ser660) [Watt et al., 2006]. Activation of protein kinase A (PKA) results in increased phosphorylation of serine sites 563, 659, and 660 [Anthonsen et al., 1998], all of which lead to increased HSL activity. Also, ERK1/2 can phosphorylate HSL at Ser600, and subsequently enhance HSL activity [Greenberg et al., 2001]. Conversely, phosphorylation of Ser565 by AMPK appears to reduce HSL activity [Garton and Yeaman, 1990].

Alterations in the expression of adipose tissue lipases have been demonstrated with obesity [D'Eon et al., 2005]; however, the effects of reduced ovarian function on ATGL and HSL signaling are unknown. There are reasons to suspect that changes in ATGL and HSL function may occur in post-menopausal females. For example, cells isolated from adipose tissue of post-menopausal women exhibit increased lipolytic rates independent of adipocyte cell size or body fat percentage [Nicklas et al., 1996; D'Eon et al., 2005]. These data suggest that the increased rates of lipolysis observed in post-menopausal women cannot be explained solely by changes in adipose tissue size. Unfortunately, very little evidence is available documenting changes in cellular signals which regulate lipolysis following a decline in ovarian function.

Thus, the purpose of this investigation was twofold: first to determine if cellular mechanisms that regulate lipid droplet function are increased in mice after surgically induced menopause and second to determine if voluntary wheel running effectively prevents increases in both visceral fat accumulation and circulating lipid in a manner similar to 17β-estradiol supplementation in ovariectomized (OVX) mice. The aim of the second component of the investigation was to determine if physical activity can be as effective as 17β-estradiol supplementation in attenuating the negative changes which occur following OVX in female mice.

METHODS

ANIMALS

Thirty-two 8-week-old C57/BL6 (Harlan) female mice were divided into four groups (SHAM, OVX, OVX-Ex, and OVX + E_2). One group of mice ($n = 12$) underwent a bi-lateral ovariectomy (OVX), a frequently used animal model of menopause [Keck et al., 2007]. Our lab has previously shown that this model decreases the levels of circulating estrogens by 70% within 48 h [Sitnick et al., 2006]. Further, previous research has shown that surgical removal of the ovaries from mice does not resulting in changes in feeding patterns [Brown, 2008; Rogers et al., 2009]. A second group ($n = 5$) was subjected to a SHAM surgery where they were anesthetized, but did not undergo ovariectomy. The third group ($n = 5$) consisted of ovariectomized mice which underwent a second surgery 1 week after ovariectomy where exogenous estrogen was introduced via implantation of a subcutaneous time-release pellet (Innovative Research, Sarasota, FL). This pellet results in the animals receiving ~40 pg/ml of 17-β estradiol per day, which is similar to levels found in the mouse during estrous [Moran et al., 2007].

Mice were given ad libitum access to water and standard rodent chow (Purina Laboratory Rodent Diet 5001: 23% protein, 4.5% fat, 6% fiber) and were housed in a temperature-controlled room on a 12 h light/dark cycle. The exercise training group (OVX-Ex) was placed in cages with voluntary running wheels, while the sedentary groups were placed in standard mouse cages. Running activity was monitored for 8 weeks with a photocell counter interfaced with a computer through customized software (Layfayette Instruments, Layfayette, IN). The 8-week time point was chosen because in preliminary experiments we found that large increases in visceral fat pad mass (i.e., parametrial fat depots) were detected at this point (data not shown). After 8 weeks all animals were sacrificed, tissue was collected and snap frozen in liquid nitrogen, and then stored at −80°C. Mice were removed from the wheel cages 24 h prior to sacrifice to account for acute effects of exercise, followed by the removal of food 4–5 h prior to sacrifice. All aspects of this study were approved by the University of Maryland Institutional Animal Care & Use Committee (IACUC) Review Board.

WESTERN BLOTTING OF ADIPOSE TISSUE

Western blotting was performed on visceral adipose tissue (i.e., parametrial fat depots). Fat was homogenized in Mueller buffer (50 mM Hepes (pH 7.4), 0.1% Triton-X100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7 \cdot H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, 50 μg/ml leupeptin, 50 μg/ml pepstatin, 40 μg/ml aprotinin, 5 mM Na_3VO_4, and 0.1% NP-40) using a mechanical homogenizer, then centrifuged for 10 min at 4°C and 13,000 rpm. The supernatant between the fat cake and the pellet was removed using a syringe and placed into a separate tube, which was spun once more under the same conditions and the supernatant was again removed. Protein concentrations were determined on the supernatant using the BCA protein assay. Equal amounts of total protein (75 μg) were resolved on 10% SDS–PAGE gels and transferred to PVDF membranes as previously described [Spangenburg et al., 2008; Wohlers et al., 2009]. Blots were visualized with Ponceau S (Sigma Chemical) to confirm equal loading of the lanes and then blocked with 5% nonfat

dry milk in Tris-buffered saline with 0.1% Tween (TBS-T). Membranes were probed with antibodies obtained from Cell Signaling (unless indicated) for p-ERK1/2 (Thr 202/Tyr 204; 1:2,000), total ERK1/2 (1:2,000), total ATGL (1:1,000), p-AMPK (Thr172; 1:1,000), p-HSL565 (1:1,000), p-HSL660 (1:1,000), total HSL (1:2,000) (kind gift of Dr. F.B. Kraemer, Stanford University), or Perilipin (1:200, Santa Cruz) in a buffer of 5% BSA in TBS-T on a rocker at 4°C overnight. Following incubation with the primary antibody, membranes were washed in TBS-T (3 × 5 min) and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (1:1,000) in 5% nonfat dry milk in TBS-T. Next, membranes were washed in TBS-T (1 × 10 min, 3 × 5 min), followed by enhanced chemiluminescence reagent (ECL) (Pierce, Rockford, IL). Membranes were visualized with a chemilumiscence imager (Syngene, Frederick, MD) and quantified with Image J software (NIH, Bethesda, MD).

IMMUNOPRECIPITATION

Two-hundred and fifty micrograms of total protein was combined with ATGL antibody (1:100 dilution) and rocked overnight at 4°C. A 25 μl aliquot of protein A-Sepharose (EZview Red Protein A Affinity Gel Beads, Sigma, St. Louis, MO) was washed repeatedly in Mueller buffer without protease inhibitors. After the last wash, the samples containing the protein and antibody complex were added to the tubes containing the protein A-Sepharose pellets. Samples were gently mixed and then rocked for 1 h at 4°C. After rocking for 1 h, samples were centrifuged at 13,000 rpm at 4°C for 5 min. The supernatant was removed and the pellet was re-suspended in 1 ml of Mueller buffer, followed by another spin of 13,000 rpm at 4°C for 5 min. This process was repeated three different times. Following the last wash, the supernatant was removed, discarded and the pellet was re-suspended in 30 μl of 4× sample loading buffer, boiled for 5 min, and centrifuged briefly. Samples were loaded onto 10% SDS–PAGE gels and transferred to PVDF membranes as previously described [Spangenburg et al., 2008; Wohlers et al., 2009]. Blots were then blocked with 5% nonfat dry milk in Tris-buffered saline with TBS-T. Membranes were probed with an rabbit polyclonal antibody specific for CGI-58 as previously described [Subramanian et al., 2004] (1:25,000, kind gift from Dr. Dawn Brasaemle, Rutgers University) or an antibody specific for total serine phosphorylation (1:1,000, Millipore). The immunoblotting protocol was the same as described above. After exposure to ECL, the membranes were stripped and re-probed for total ATGL as described above. All quantified data were normalized to total ATGL expression.

NEFA AND GLYCEROL LEVELS

Non-esterified fatty acids (NEFA) were measured in mouse serum using a colorimetric assay (Wako Diagnostics, Richmond, VA). Glycerol levels were determined in mouse serum or media using a free glycerol determination kit (Sigma–Aldrich, St. Louis, MO).

ISOLATED ORGAN BATH EXPERIMENTS

In a second set of animals (SHAM n = 5; OVX n = 5), organ bath experiments were performed on isolated visceral adipose tissue (i.e., parametrial fat depots). The visceral adipose tissue was carefully dissected and placed in an organ bath containing oxygenated Ringers media (95% O_2; 5% CO_2). After 30 min, half of the adipose tissue mass was removed and frozen. In addition, 1 ml of the media was removed and frozen for glycerol analysis. The remaining adipose tissue was washed with media and then exposed to fresh media containing the MEK inhibitor PD98059 (30 μM) for another 30 min. Previous data from our lab demonstrated that PD98059 can effectively inhibit ERK1/2 activation [Spangenburg and Booth, 2002]. The remaining tissue was frozen and 1 ml of media was also frozen for subsequent glycerol analysis. All glycerol measurements from each condition were normalized to the total protein content from that specific isolated adipose tissue.

STATISTICAL ANALYSIS

All data are expressed as means ± SEM. Statistical significance was determined using *t*-tests with Sigma Stat statistical analysis software (Systat Software, Inc., San Jose, CA). A *P*-value of <0.05 was considered significant. A post hoc statistical power analysis was conducted based on our SHAM and OVX fat mass, glycerol, and total ATGL protein content data. Using five mice per group we had between 69% and 96% statistical power (alpha = 0.05) to detect differences between the groups.

RESULTS

ANATOMICAL CHARACTERISTICS

The body weight of the sedentary OVX animals was significantly greater than the SHAM animals by 9.8%; however, no differences were detected in absolute liver, heart, or skeletal muscle masses (Table I). The visceral fat mass in the sedentary OVX group was significantly greater than all other groups by ~300% (Table I). The visceral fat mass of OVX-Ex mice was significantly lower than sedentary OVX mice ($P < 0.05$); however, still significantly greater than the SHAM group ($P < 0.05$). When fat mass was normalized to

TABLE I. Anatomical Characteristics for SHAM, OVX, OVX + Ex, and OVX + E_2 Groups

	Body weight (g)	Fat mass (g)	Fat mass/body weight	Liver (g)	Heart (g)	TA (g)	Quad (g)
SHAM	22.94 ± 0.57	0.27 ± 0.06	0.011 ± 0.002	0.904 ± 0.06	0.102 ± 0.002	0.039 ± 0.0005	0.148 ± 0.005
OVX	25.20 ± 0.96*	0.83 ± 0.14*	0.03 ± 0.005*	0.940 ± 0.07	0.105 ± 0.007	0.037 ± 0.0009	0.149 ± 0.009
OVX Ex	23.85 ± 0.53	0.46 ± 0.09*,$	0.02 ± 0.003	0.939 ± 0.06	0.106 ± 0.002	0.036 ± 0.001	0.142 ± 0.007
OVX + E_2	23.72 ± 0.43	0.27 ± 0.07	0.01 ± 0.003	1.025 ± 0.05	0.104 ± 0.004	0.04 ± 0.003	0.150 ± 0.004

Values are presented as means ± SEM.
*Significantly different from SHAM group ($P < 0.05$).
$Significantly different from OVX group ($P < 0.05$).

TABLE II. Fasted Glycerol and NEFA Levels From SHAM, OVX, OVX + Ex, and OVX + E_2 Groups

	SHAM	OVX	OVX + Ex	OVX + E_2
Glycerol (mg/ml)	304.43 ± 23.61	467.33 ± 52.00*	484.25 ± 102.78#	373.45 ± 51.50
NEFA (mmol/L)	0.424 ± 0.0057	0.649 ± 0.0101*	0.555 ± 0.0133*	0.411 ± 0.0088
Glucose (mg/dl)	188.5 ± 14.48	221.42 ± 9.09*	176.42 ± 19.40$	177.1 ± 20.07$

Values are presented as means ± SEM.
*Significantly different from SHAM and OVX + E_2 groups ($P < 0.05$).
$Significantly different from OVX group ($P < 0.05$).
#Compared to SHAM ($P = 0.06$).

body weight, only the sedentary OVX mice differed significantly from the SHAM mice ($P < 0.05$). OVX mice with voluntary wheel access ran 2448.80 ± 157.85 m/day, which is consistent with previously published data [Gorzek et al., 2007]. Supplementation with 17β-estradiol prevented any increase in visceral fat mass of the OVX animals when compared to the SHAM animals (Table II).

NEFA AND GLYCEROL LEVELS

Fasted NEFA levels were significantly elevated in the OVX and OVX + Ex groups compared to the SHAM and OVX + E_2 groups. Glycerol levels were significantly elevated in the sedentary OVX mice as compared to SHAM mice. However, glycerol levels of the OVX + Ex group were elevated and reached levels close to statistical significance when compared to the SHAM group ($P = 0.06$).

ATGL PROTEIN CONTENT AND INTERACTION WITH CGI-58

To understand the effect of ovarian hormones on cellular signaling in visceral adipose tissue, we measured indices of ATGL and HSL activation. Total ATGL protein content was elevated in OVX and OVX + Ex mice as compared to SHAM (Fig. 1), while ATGL content in OVX + E_2 mice was not significantly different from SHAM mice. Using a novel immunoprecipitation assay we found increased ATGL–CGI-58 interaction in the OVX and OVX + Ex mice compared to SHAM. OVX + E_2 mice exhibited levels of ATGL–CGI-58 interaction that were significantly lower than the OVX mice and not different from the SHAM mice (Fig. 2). Further, we found no differences in total serine phosphorylation of immunoprecipated ATGL between any groups (data not shown).

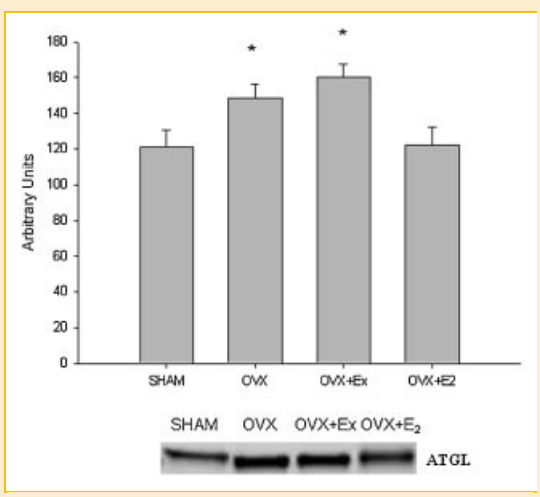

Fig. 1. Ovariectomy increased total ATGL protein content in the visceral fat pad, but 17β-estradiol supplementation prevented the increase. Example blots of ATGL are shown beneath the graph. * Indicates significantly different from SHAM ($P < 0.05$).

HSL PROTEIN CONTENT AND PHOSPHORYLATION

Next, we sought to determine if changes in HSL phosphorylation were detectable in visceral fat isolated from these animals (Fig. 3A). Minimal phosphorylation was detected, with no quantitative differences in phosphorylation of HSL at Ser659/660, a site which is phosphorylated by PKA and thought to be necessary for complete activation of HSL (Fig. 3B; quantitative data not shown). We detected minor alterations in AMPK phosphorylation (Thr172) in visceral fat; however, these differences did not correlate to changes in HSL565 phosphorylation (Fig. 3B; quantitative data not shown). This suggests that the Ser565 site on HSL may also be phosphorylated by a kinase other than AMPK [Garton and Yeaman, 1990].

ALTERATIONS IN ERK1/2 PHOSPHORYLATION

A previous investigation has documented that ERK1/2 phosphorylates HSL at Ser600 and results in a significant increase in HSL

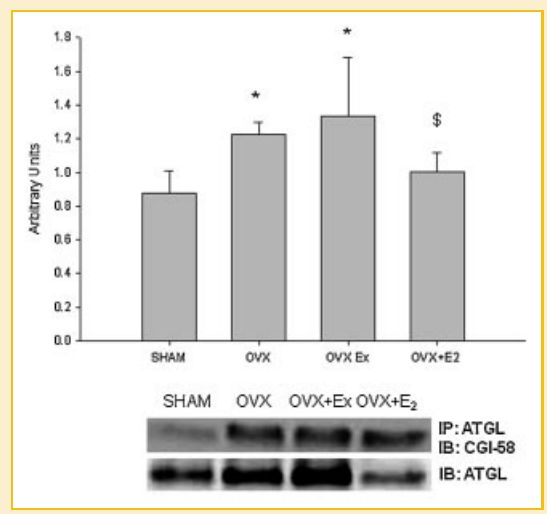

Fig. 2. ATGL–CGI-58 interaction was elevated in OVX mice, but this increase was attenuated in OVX + E_2 mice. The blot was stripped and re-probed for ATGL. All CGI-58 data were normalized to ATGL protein content. Example blots of CGI-58 and ATGL are shown beneath the graph. * Indicates significantly different from SHAM ($P < 0.05$). $ Indicates significantly different from OVX ($P < 0.05$).

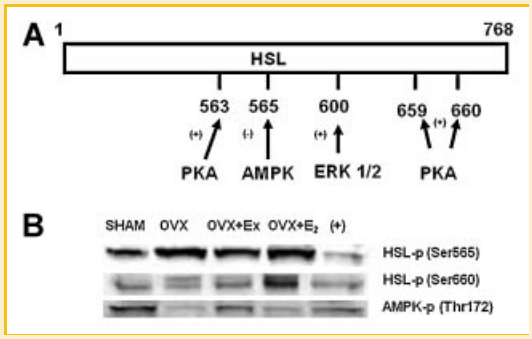

Fig. 3. A,B: Ovariectomy had little effect on HSL phosphorylation in visceral fat at the Ser565 or Ser600 residues. A: Regulation of HSL. (+) Indicates activation via phosphorylation. (−) Indicates inactivation via phosphorylation [adapted from Watt et al., 2006]. B: No significant changes were detected in HSL565 or HSL660 phosphorylation in any sample.

activity [Souza et al., 2003]. In our samples, significant differences were detected between the SHAM and sedentary OVX mice, as well as between the sedentary OVX and OVX + E_2 mice in normalized ERK1 (phosphorylated/total) (Fig. 4). Significant differences were also detected between the SHAM and sedentary OVX mice, as well as between the SHAM and OVX + E_2 mice in normalized ERK2 (phosphorylated/total) (Fig. 4). Unfortunately, it is not possible to determine if these differences in ERK1/2 activation resulted in altered HSL phosphorylation at serine residue 600 since there are no available antibodies to measure phosphorylation changes at this site.

GLYCEROL LEVELS AND ERK1/2 PHOSPHORYLATION WITH MAPK INHIBITOR APPLICATION

Thus, to determine if activation of ERK1/2 is critical for increased glycerol release by the adipose tissue of the OVX animals, we

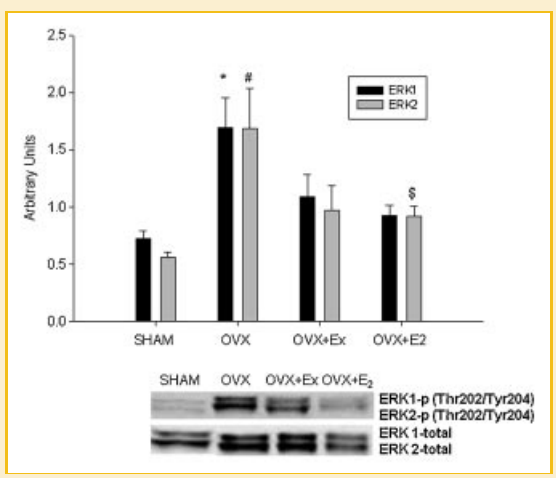

Fig. 4. Normalized phosphorylation of ERK1/2 was elevated in OVX mice and attenuated by both voluntary wheel running and 17β-estradiol supplementation. Example blots of ERK1/2 are shown beneath the graph. * Indicates significantly different from all other groups ($P < 0.05$). # Indicates significantly different from SHAM ($P < 0.05$). $ Indicates significantly different from OVX ($P < 0.05$).

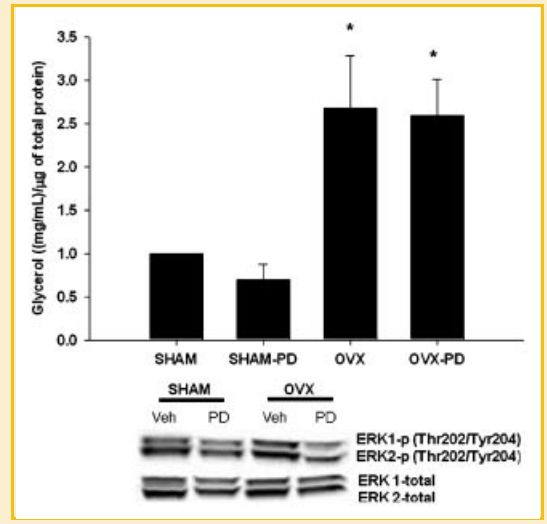

Fig. 5. Pharmacological inhibition of ERK1/2 by MEK inhibitor PD98059 did not reduce levels of glycerol released from the visceral adipose tissue isolated from OVX mice. Example blots of ERK1/2 are shown beneath the graph. * Indicates significantly different from SHAM and SHAM-PD ($P < 0.05$).

isolated visceral adipose tissue from SHAM and OVX animals for organ bath experiments. The adipose tissue was divided and placed in oxygenated media. To achieve baseline values, the tissue was incubated for 30 min, after which a portion of the adipose tissue and media was removed and frozen. The remaining adipose tissue was exposed to fresh media supplemented with the MEK inhibitor PD98059 (30 μM). Glycerol levels were significantly elevated in the media which incubated visceral adipose tissue from the OVX mice compared to the media that incubated visceral adipose tissue from the SHAM mice (Fig. 5). However, even though the concentration of PD98059 was sufficient to reduce ERK1/2 phosphorylation, the drug did not decrease glycerol levels in either group (Fig. 5).

PERILIPIN PROTEIN CONTENT

Perilipin plays a critical role in lipolysis by regulating lipase function at the lipid droplet [Shen et al., 2009]. Perilipin protein content was significantly decreased in the OVX and OVX + Ex groups compared to SHAM. Animals supplemented with 17β-estradiol had significantly greater perilipin protein content compared to OVX and OVX + Ex animals. However, perilipin protein content was still significantly lower in the OVX + E_2 group than in the SHAM group (Fig. 6).

DISCUSSION

The decline in ovarian hormone levels during menopause has been associated with changes in body fat and body composition [Panotopoulos et al., 1997; Schneider et al., 2006; Dubnov-Raz et al., 2007; Rosano et al., 2007], and results in increased basal (i.e., non-stimulated) lipolysis [Ferrara et al., 2002]. The data presented here demonstrate that reduced ovarian function in OVX mice results

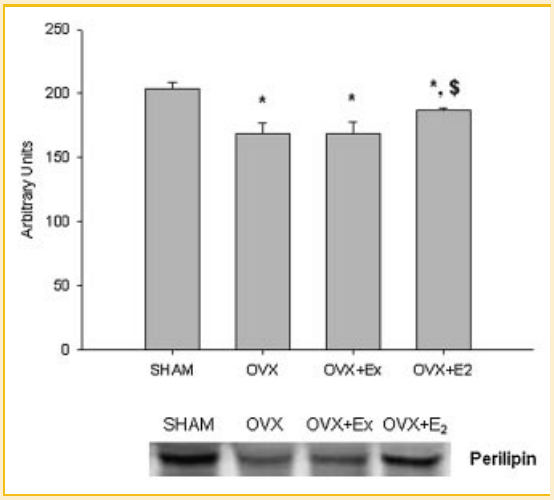

Fig. 6. Perilipin protein content was decreased in OVX and OVX-Ex animals and partially restored in OVX + E_2 mice. Example blots of total perilipin are shown beneath the graph. * Indicates significantly different from SHAM ($P < 0.05$). $ Indicates significantly different from OVX ($P < 0.05$).

in significant elevation of serum glycerol and NEFA levels, as well as increased visceral (i.e., parameterial) fat mass compared to SHAM mice. Thus, qualitatively the OVX mice present with similar metabolic changes as described previously in post-menopausal women [Schneider et al., 2006]. The increased glycerol and NEFA levels in the OVX mice were associated with increased ATGL–CGI-58 interaction and reduced perilipin protein content in the visceral fat as compared to the SHAM animals. These changes were attenuated with 17β-estradiol supplementation, but not with voluntary wheel running. Surprisingly, minimal differences in HSL phosphorylation were detected between any of the groups. In spite of increases in ERK1/2 phosphorylation in the OVX animals, it does not appear that increased ERK1/2 activity contributes to the enhanced glycerol release by the adipose tissue. These data suggest that increases in serum glycerol and NEFA in the OVX animals as compared to the SHAM animals may be due to increased ATGL signaling and reduced perilipin protein content in the visceral adipose tissue.

Similar to previous studies [Gambacciani et al., 2001; Jensen et al., 2003], we found that 17β-estradiol supplementation prevented the increases in visceral fat mass, and serum glycerol and NEFA levels in OVX mice. D'Eon et al. [2005] found increased levels of serum FFA following fasting in OVX mice, which were successfully attenuated with 17β-estradiol supplementation. However, no SHAM group was presented in their data making it unclear how the FFA values compared to mice with intact ovaries. Further, our data agree with D'Eon et al. who found increased glycerol release in fat isolated from OVX mice compared to 17β-estradiol supplemented OVX mice. Also, in line with other studies, fat mass accumulation was attenuated in the OVX mice supplemented with 17β-estradiol [Gray and Wade, 1981; Pelleymounter et al., 1999; Shinoda et al., 2002; McCormick et al., 2004]. OVX mice exposed to voluntary running wheels exhibited significantly lower visceral fat masses compared to sedentary OVX animals. However, voluntary running in the OVX mice did not prevent the changes in serum NEFA or glycerol levels. It should be noted that SHAM female mice will run an average of 7 km/day (data not shown). In contrast, the OVX mice in this experiment ran substantially less (~2 km/day). Therefore, it is possible that the distances run by the OVX animals were not enough to affect mechanisms regulating NEFA and glycerol release from adipose tissue. This reduced running distance by the OVX mice has also been previously documented by other labs [Gorzek et al., 2007]. It is unclear if the documented changes in cellular signaling in the adipose tissue from the OVX mice would be attenuated or abolished if the OVX mice ran distances similar to the SHAM mice. Supplementation with 17β-estradiol partially returns voluntary running activity, with mice running on average 4–6 km/day (data not shown). Based on these data, 17β-estradiol appears to have a more potent effect than voluntary wheel running on cellular signaling mechanisms in the visceral fat of OVX mice.

At a cellular level, TAG hydrolysis is regulated by several lipases, most notably ATGL and HSL, which combined account for more than 95% of TAG hydrolase activity in white adipose tissue [Schweiger et al., 2006]. Total ATGL protein content was elevated in adipose tissue of OVX animals and attenuated in animals supplemented with 17β-estradiol, but not with voluntary exercise. The inability of voluntary exercise to affect total ATGL protein content agrees with Huijsman et al. [2009] who found no change in adipose tissue ATGL protein content in mice following an endurance training protocol. Previous publications have suggested ATGL can be phosphorylated at several serine residues [Zimmermann et al., 2004], however, we found no differences between any groups in total serine phosphorylation of ATGL. Full activation of ATGL is dependent upon interaction with another protein, CGI-58 [Lass et al., 2006]. We found that the OVX animals demonstrated a significant increase in ATGL–CGI-58 interaction, which was associated with increases in serum glycerol and NEFA levels. As has been shown in cell culture studies, this interaction of ATGL and CGI-58 appears to be a critical link contributing to increases in basal lipolysis of TAG [Schweiger et al., 2006]. The increase in ATGL–CGI-58 interaction in the OVX mice was not attenuated by exercise, but was reduced by 17β-estradiol supplementation, corresponding with the changes in glycerol and NEFA levels.

Perilipin is the major protein associated with the lipid droplet in adipocytes and plays a critical role in the regulation of adipose tissue metabolism by controlling lipase access to stored TAGs [Shen et al., 2009]. Perilipin null mice have constitutively high lipolytic rates with little activation of HSL in the adipose tissue, confirming a protective role for perilipin in adipocytes [Saha et al., 2004]. The phenotype of the perilipin null mouse is remarkably similar to the OVX mouse. Our OVX and OVX + Ex mice, which exhibit increased serum glycerol and NEFA levels, also express significantly lower perilipin protein content in visceral adipose tissue. This suggests that loss of perilipin in the adipose tissue of OVX mice is associated with increased TAG breakdown. In addition, voluntary exercise in the OVX mice did not prevent the decrease in perilipin protein content in the visceral adipose tissue, while 17β-estradiol supplementation completely attenuated the decrease. Previous research indicates that voluntary exercise does not change perilipin protein content in the mesenteric fat pad [Chapados et al., 2008]; however, 17β-estradiol

supplementation does prevent the loss of perilipin [D'Eon et al., 2005].

Following the breakdown of TAG to DAG by ATGL, HSL continues the process of lipolysis, breaking DAG to MAG. We found little difference between our groups in HSL phosphorylation at the Ser565 or Ser559/660 residues, suggesting that HSL is not contributing to differences in circulating NEFA and glycerol levels in the OVX mice. This result has also been shown in the perilipin null mice, where very little activation of HSL was detected in spite of increased rates of glycerol release from adipose tissue [Saha et al., 2004]. Further, we found differences in AMPK phosphorylation did not correspond with differences in HSL565 (Fig. 3B), suggesting that a factor other than AMPK is altering the phosphorylation status of Ser565 in the visceral fat from our animals. Previous studies have shown that ERK1/2 also enhances HSL activity [Greenberg et al., 2001; Huang et al., 2004] by phosphorylating HSL at Ser600, and subsequently enhancing lipolysis [Souza et al., 2003]. ERK1/2 phosphorylation was significantly elevated in sedentary OVX animals, suggesting activation of ERK1/2 may increase TAG hydrolysis. As expected, application of PD98059 to adipose tissue incubated in a bath system decreased phosphorylation of ERK1/2, however, glycerol levels in the solution remained equivalent to those of the non-treated media. Therefore, in the OVX mice, activation of ERK1/2 is unlikely to be responsible for the elevated glycerol levels.

Estrogen has been shown to have an effect on adipocyte size, with estrogen treatment resulting in decreased adipocyte diameter [D'Eon et al., 2005]. Therefore, it is possible to hypothesize that changes in adipocyte cell size in the visceral fat of OVX mice may contribute to the observed changes in protein content. In opposition, previous data have suggested that increased rates of lipolysis detected in post-menopausal women are not related to adipocyte cell size [Nicklas et al., 1996]. Further studies will be conducted to determine if changes in fat cell size are occurring and contributing to the observed alterations in lipolytic protein content.

In addition, insulin plays a strong role in the regulation of lipolysis. Specifically, insulin decreases lipolytic rates in adipose tissue by increasing phosphodiesterase-3B (PDE-3B) activity [Smith et al., 1991; Eriksson et al., 1995]. PDE-3B degrades cAMP levels resulting in the removal of a major stimulus necessary for the complete activation of lipolysis. Previous studies have suggested that ovariectomy results in peripheral insulin resistance [Saengsirisuwan et al., 2009], which could contribute to the increased basal levels of glycerol and NEFA in the sedentary OVX animals. Therefore, it is possible that alterations in circulating insulin levels and/or insulin receptor sensitivity may be contributing to the changes in lipolysis which occur following ovariectomy. While insulin sensitivity was not measured in this set of mice, we are currently working on experiments to determine the role of insulin in the regulation of lipolysis in the OVX mouse model.

The data presented here demonstrate for the first time that reduced ovarian function results in changes in signaling proteins which regulate TAG hydrolysis. Specifically, we found increased interaction of ATGL–CGI-58 and reduced perilipin protein content in the visceral fat of OVX mice. These findings provide evidence of cellular mechanisms contributing to elevated glycerol and NEFA levels in female mice following ovariectomy, and further suggest that low levels of physical activity may not effectively prevent these changes. However, this should not be misconstrued that physical activity is not beneficial when ovarian hormone levels are decreased. It is well documented that endurance exercise training results in enhanced lipid oxidation by peripheral tissues and thus even though exercise failed to attenuate the increased lipolysis in OVX mice, it is likely that various peripheral organs (i.e., skeletal muscle) gained an improved ability to oxidize the excess lipid. Clearly, exercise provides numerous other documented benefits and should still be considered as part of a critical intervention for post-menopausal women.

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