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17β-Estradiol and Anti-estrogen ICI:Compound 182,780 Regulate Expression of Lipoprotein Lipase and Hormone-Sensitive Lipase in Isolated Subcutaneous Abdominal Adipocytes

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We sought to investigate the influence of 17β-estradiol (E_2) on key enzymes of lipogenesis and lipolysis in subcutaneous (SC) abdominal adipocytes isolated from women. In addition, we wished to determine the influence of an anti-estrogen, ICI: compound 182,780 (anti-E), known to act via the estrogen receptor (ER), alone and in combination with E_2. Adipose tissue was obtained from 17 women undergoing elective surgery, with a mean age of 47 years (range, 34 to 62), mean weight of 65.4 kg (range, 58.1 to 75.0), and mean body mass index (BMI) of 25 kg/m^2 (range, 22 to 27). Isolated adipocytes were treated with varying doses of E_2, anti-E, or E_2 in combination with anti-E 10^{-8} mol/L for 48 hours. Following treatment, proteins were extracted and the effects on lipogenesis and lipolysis were assessed, using Western blotting to determine the relative expression of the key enzymes of these processes, lipoprotein lipase (LPL; 56 kd), and hormone-sensitive lipase (HSL; 84 kd), respectively. Glycerol release into the medium was also measured as an index of lipolytic activity. The protein expression studies demonstrated that E_2 altered expression of LPL relative to control, with the highest dose significantly reducing LPL expression and the lower doses significantly increasing LPL expression (mean protein expression relative to control ± SE): E_2 10^{-12} mol/L, 1.79 ± 0.16 ($P < .001$); E_2 10^{-7} mol/L, 0.56 ± 0.08 ($P < .05$). In contrast, HSL expression was increased relative to control at the higher doses of E_2 but was not significantly altered relative to control at the lower doses: E_2 10^{-12} mol/L, 1.02 ± 0.14 ($P > .05$); E_2 10^{-7} mol/L, 1.55 ± 0.17 ($P < .01$). Anti-E 10^{-8} mol/L alone reduced LPL protein expression relative to control ($P < .05$) and increased HSL protein expression relative to control ($P > .05$). In combination with E_2 10^{-7} mol/L, anti-E 10^{-8} mol/L did not abrogate the inhibitory effect on LPL expression relative to control ($P < .05$). Furthermore, E_2 10^{-7} mol/Lin combination with anti-E 10^{-8} mol/L, displayed a stimulatory effect on HSL expression relative to control ($P < .01$). Glycerol release studies following the higher doses of E_2, and also following E_2 10^{-7} mol/L in combination with anti-E 10^{-8} mol/L, provided support for the HSL protein expression studies. We conclude that the highest concentration of E_2 (10^{-7} mol/L) significantly reduced LPL expression relative to control, while the lower concentrations significantly increased LPL expression relative to control. The highest concentration of E_2 also significantly increased both HSL expression and glycerol release relative to control. The effects of anti-E suggest that the in vitro effects of E_2 on lipogenesis and lipolysis occur, at least in part, through a receptor-mediated pathway. In addition, as recently observed in other tissues, ICI:compound 182,780 does not appear to behave as a pure anti-estrogen in isolated human adipocytes.

OBESITY IS A widely accepted cause of increased morbidity and mortality, with an increased prevalence of chronic conditions such as coronary heart disease, type 2 diabetes, dyslipidemia, and hypertension.[1] The distribution of body fat is of particular relevance, with centrally located fat (abdominal obesity) conferring a greater risk.[2,3] Vague's epidemiological work suggested sex steroids as important candidate factors in determining body fat distribution, with a distinct gender difference proposed.[4] It is now widely accepted that males tend towards a central fat distribution (android obesity), while premenopausal females tend towards a gluteo-femoral fat distribution (gynoid obesity). Furthermore, sex steroids may explain why premenopausal women have a lower prevalence of cardiovascular disease than men, with the relative cardiovascular risk in women increasing following menopause.[5] Clinical prospective studies have suggested that combined hormone-replacement therapy (HRT) may prevent or limit the change in

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fat distribution from gluteo-femoral to abdominal commonly observed in women following menopause.[6,7] Supporting evidence comes from work investigating cross–sex hormone administration to transsexuals, with regional sex differences in body fat distribution being influenced by the sex steroids present.[8]

The number and size of adipocytes in an area of adipose tissue determine the mass of that depot. In humans, the number of adipocytes present in a depot varies between anatomical sites and also between males and females.[9] Adipocyte size is determined by the net balance of lipogenesis and lipolysis[10] and while size varies with anatomical site and sex,[9] a critical cell size exists, beyond which an increase in adipocyte number is seen.[11] Lipogenesis involves the incorporation of free fatty acids (FFA) into the adipocyte for storage as triglyceride (TG), with many enzymes regulating this process. Since the major determinant of lipogenesis in adipose tissue is the re-esterification of FFA, lipoprotein lipase (LPL) is of paramount importance.[12] The hydrolysis of TG stored in adipocytes to FFA and glycerol occurs by the process of lipolysis, with the rate-limiting enzyme being hormone-sensitive lipase (HSL).[13] Human studies suggest that lipogenesis and lipolysis vary by anatomical site, with these processes being influenced by catecholamines, insulin, growth hormone, glucocorticoids, sex steroids, thyroid hormones, and acylation-stimulating protein.[10,14]

17β-Estradiol (E_2) has been shown to regulate adipose tissue mass by increasing adipocyte number through effects on proliferation[15,16] and differentiation.[16] E_2 may also regulate adipose tissue mass through effects on adipocyte size. Animal studies have shown both E_2 and 2-fluoro-estradiol to reduce markers of lipogenesis[17-20] and increase markers of lipolysis.[19-21] Some,[22,23] but not all,[24] studies involving humans have suggested a similar relationship between E_2 and LPL activity in gluteal adipose tissue. Clinical studies of oral estrogen-replacement therapy (ERT) suggest an improvement in the plasma lipid profile, although any improvement is marred by an increase in serum TG suggestive of increased lipolysis.[25,26]

We investigated the influence of E_2 on key enzymes of lipogenesis and lipolysis in isolated adipocytes obtained from the subcutaneous (SC) abdominal adipose tissue of women. In addition, using the anti-estrogen, ICI:compound 182,780 (anti-E), which is known to act via the estrogen receptor (ER), both alone and in combination with E_2, we investigated the mechanism through which E_2 acts in human adipose tissue.

MATERIALS AND METHODS

Subjects

SC abdominal adipose tissue was obtained from 17 women undergoing elective abdominal surgery, with the majority of cases being performed for cosmetic reasons. The mean age for the group was 47 years (range, 34 to 62), mean weight was 65.4 kg (range, 58.1 to 75.0), and mean body mass index (BMI) was 25 kg/m^2 (range, 22 to 27). Subjects using endocrine therapy such as corticosteroids, thyroxine, and either the oral contraceptive pill or HRT were excluded, as were those on lipid-lowering medication. Adipose tissue was collected with the approval of the South Birmingham Health Authority Ethics Committee.

Tissue Culture

Fresh adipose tissue (1 to 2 g) was collected and washed with phosphate-buffered saline (PBS) (Sigma, Poole, UK) containing penicillin (100 U/mL) (Gibco, Paisley, UK) and streptomycin (100 μg/mL) (Gibco). Visible blood vessels and connective tissue were removed prior to digestion with collagenase class 1 (2 mg/mL, Worthington Biochemical Corp, Twyford, UK) made up in 1x Hank's balanced salt solution (HBSS) (Gibco). Digestion time varied between 45 and 60 minutes, depending on the tissue obtained, in a shaking water bath (100 cycles/min) at 37°C. The digested tissue was filtered through double-layered cotton mesh prior to separation of the adipocytes by centrifugation at 2,000 rpm for 5 minutes.

Isolation and Treatment of Mature Adipocytes

Following centrifugation, the supernatant containing the mature adipocytes was removed and washed in phenol red-free Dulbecco's modified Eagle's medium (DMEM/F12) (Gibco) before centrifugation at 1,000 rpm for 30 seconds. Compacted 1-mL aliquots of adipocytes (500,000 cells) were placed into flasks containing 5 mL of phenol red-free DMEM/F12, with penicillin (100 U/mL), streptomycin (100 μg/mL), and transferrin (5 μg/mL) (Sigma). The adipocytes were treated with E_2 10^{-12} mol/L to 10^{-7} mol/L alone (Sigma, UK), anti-E 10^{-9} mol/L to 10^{-7} mol/L alone (Tocris Cookson Corp, Bristol, UK), and E_2 10^{-12} mol/L to 10^{-7} mol/L in combination with anti-E 10^{-8} mol/L. Control were adipocytes maintained in flasks containing culture medium alone. Adipocyte viability was assessed as previously described using trypan blue (Sigma).[27]

Protein Extraction and Collection of Medium

Following incubation at 37°C for approximately 48 hours, the flask contents were removed and centrifuged at 2,000 rpm for 5 minutes. The medium was collected and stored at −70°C for subsequent analysis. The adipocyte protein was extracted using 4% sodium dodecyl sulfate (SDS) (Bio-Rad, UK) and heated for 4 to 6 hours before the extracted proteins were also stored at −70°C. Due to the large number of treatments involved, it was not always possible to obtain sufficient material for all of the treatments to be studied in each patient.

Protein Assay

The quantity of protein in each sample was determined using a colourimetric Detergent Compatible protein assay kit (Bio-Rad, UK). The protein samples and bovine serum albumin (BSA 1 mg/mL) (Sigma) as a protein standard, were mixed with the dye concentrate and incubated at 25°C for 15 minutes. The absorbencies were read within 30 minutes at a wavelength of 655 nm on a spectrophotometer. A protein standard curve was constructed and used to calculate the amount of protein required.

Western Blotting

Equal amounts of protein for each treatment and control (10 to 100 μg) were prepared for analysis using Western blotting. Prestained molecular weight markers were used as standards (Amersham Pharmacia Biotech, Little Chalfont, UK). The protein samples were heated to 95°C in a sample buffer containing dithiothreitol 100 nmol/L (Sigma), separated by SDS-polyacrylamide gel electrophoresis (4.5% stacking gel, pH 6.8; 10% resolving gel, pH 8.8), and electrophoretically transferred onto polyvinylidene fluoride (PVDF) microporous membranes (Immobilon-P transfer membrane; Millipore, Bedford, MA). The membranes were blocked in 10% (wt/vol) non-fat milk powder (Marvel, Spalding, UK) for 1 hour before being isolated overnight at 4°C with primary antibody raised against LPL (56 kd) (1

in 5,000 with BSA 0.05%) (Research Diagnostics Inc, Flanders, NJ) or HSL (84 kd) (1 in 500).[28] The membranes were then isolated with secondary antibody (anti-mouse and anti-rabbit for LPL and HSL, respectively; The Binding Site Ltd, Birmingham, UK) at room temperature, before chemiluminescent detection, using ECL Plus for LPL and ECL for HSL (Amersham Pharmacia Biotech). The radiographs were quantified using Windows:Gelbase/Gelblot (UVP Ltd, Cambridge, UK).

Glycerol Assay

The media were analyzed in triplicate using a colourimetric method (Randox Laboratories, Crumlin, UK). The assay measures the concentration of glycerol and thus provides a measure of the rate of lipolysis. A standard curve was plotted from a glycerol standard solution and used to convert wavelength readings into glycerol concentrations (μmol/L).

Statistical Analysis

Statistical analysis was performed using analysis of variance (Statview; Abacus Concepts Inc, Berkeley, CA). Results are expressed relative to control for expression of LPL and HSL, although for glycerol, absolute levels are given. Data in the text and figures are presented as the mean ± SE. The threshold of significance was taken at $P < .05$.

RESULTS

LPL and HSL Protein Expression Studies

Effect of E_2. Increasing concentrations of E_2 altered LPL protein expression relative to control, with the highest dose ($E_2 10^{-7}$ mol/L) significantly reducing LPL protein expression and the lower doses increasing LPL protein expression; mean protein expression relative to control ± SE: E_2 10^{-12} mol/L, 1.79 ± 0.16 ($P < .001$); E_2 10^{-7} mol/L, 0.56 ± 0.08 ($P < .05$) (Fig 1A).

In contrast, the higher concentrations of E_2 increased HSL protein expression relative to control, while the lower doses did not significantly alter protein expression relative to control: $E_2 10^{-12}$ mol/L, 1.02 ± 0.14 ($P > .05$); $E_2 10^{-7}$ mol/L, 1.55 ± 0.17 ($P < .01$) (Fig 1B).

Effect of anti-E. Expression of LPL protein was reduced relative to control by anti-E treatment at 10^{-9} mol/L, 0.59 ± 0.10 ($P < .01$), and at 10^{-8} mol/L, 0.68 ± 0.10 ($P < .05$), but was not significantly altered at 10^{-7} mol/L (Fig 2A).

Expression of HSL protein was nonsignificantly increased relative to control by anti-E treatment at the higher concentrations: 10^{-8} mol/L, 1.08 ± 0.18 ($P > .05$); 10^{-7} mol/L, 1.43 ± 0.15 ($P > .05$) (Fig 2B).

Effect of E_2 in combination with anti-E. In combination with E_2, anti-E 10^{-8} mol/L did not abrogate the inhibitory effect on LPL protein expression at 10^{-7} mol/L: 0.62 ± 0.05 ($P < .05$). At the lower concentrations of E_2 in combination with anti-E 10^{-8} mol/L, LPL protein expression was not significantly altered relative to control (Fig 3A).

The stimulatory effect of E_2 10^{-7} mol/L on HSL protein expression appeared greatest when given in combination with anti-E 10^{-8} mol/L: 1.93 ± 0.31 ($P < .01$). At the lower concentrations of E_2 in combination with anti-E 10^{-8} mol/L, HSL protein expression was not significantly altered relative to control (Fig 3B).

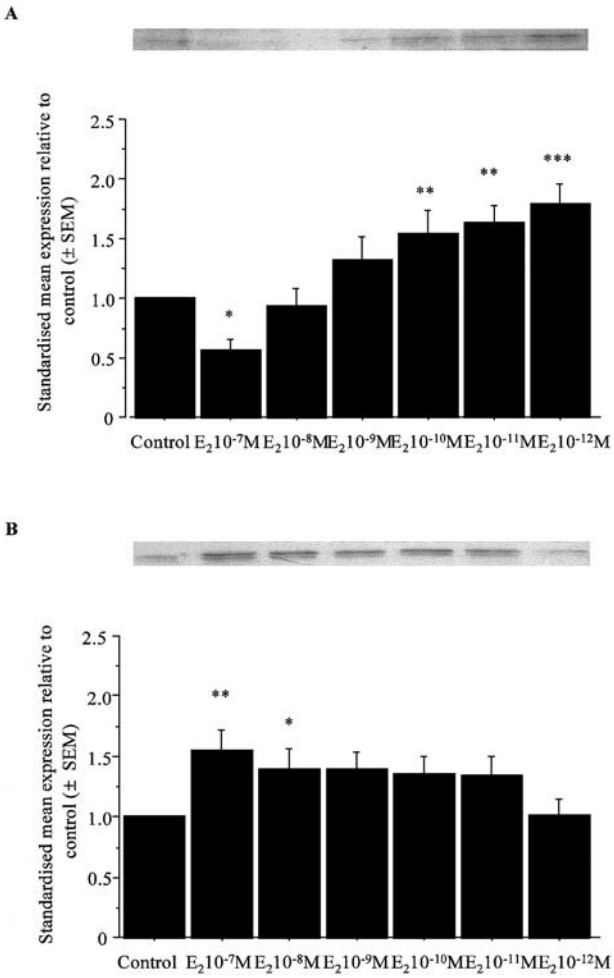

Fig 1. Mean relative protein expression (± SE) of E_2 treatments from 10^{-12} mol/L (M) to 10^{-7} mol/L compared to control for (A) LPL (n = 5) and (B) HSL (n = 5) in isolated mature SC adipocytes (*$P < .05$, **$P < .01$, ***$P < .001$). A representative Western blot is shown for both of the graphs.

Glycerol Release Studies

All doses of E_2 resulted in significantly higher glycerol release into the medium compared to control: control, 353.2 μmol/L ± 17.7; E_2 10^{-12} mol/L, 452.5 μmol/L ± 26.7 ($P < .01$); E_2 10^{-7} mol/L, 477.7 μmol/L ± 30.3 ($P < .01$) (Table 1). These data support the HSL protein expression studies at the higher doses of E_2. Glycerol release into the medium following anti-E was not significantly different to control at any dose (Table 2). Glycerol release into the medium following E_2 10^{-7} mol/L with anti-E 10^{-8} mol/L was significantly increased compared to control: E_2 10^{-7} mol/L/anti-E 10^{-8} mol/L,: 433.8 μmol/L ± 41.9 ($P < .01$), again validating the HSL expression studies. No significant effect on glycerol release was detected with anti-E 10^{-8} mol/L in combination with the lower E_2 doses (Table 2).

DISCUSSION

Human epidemiological and prospective clinical studies suggest estrogen deficiency is associated with the development of

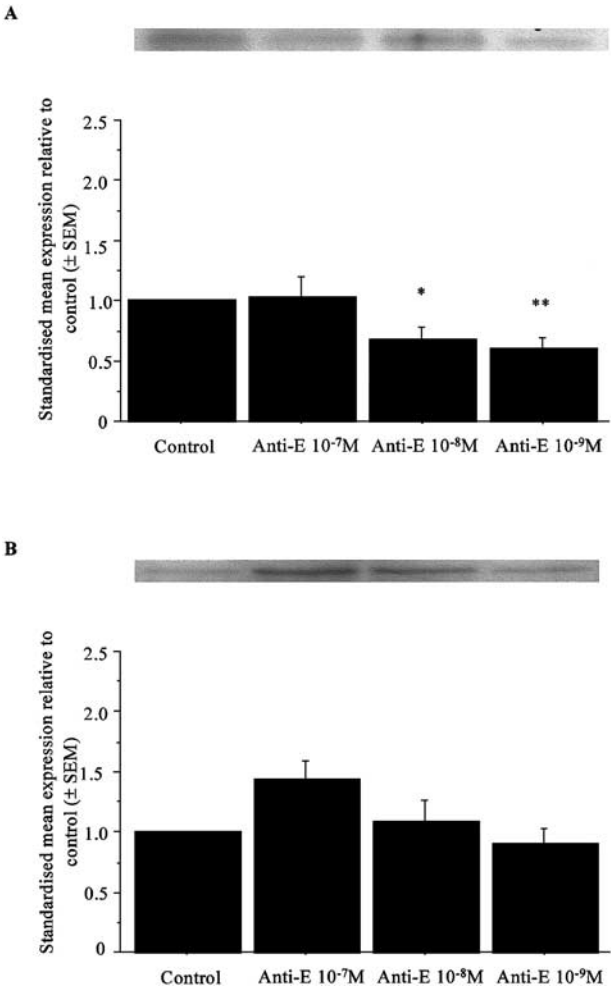

Fig 2. Mean relative protein expression (± SE) of anti-E treatments from 10^{-9} mol/L (M) to 10^{-7} mol/L compared to control for (A) LPL (n = 5) and (B) HSL (n = 5) in isolated mature SC adipocytes (*P < .05, **P < .01). A representative Western blot is shown for both of the graphs.

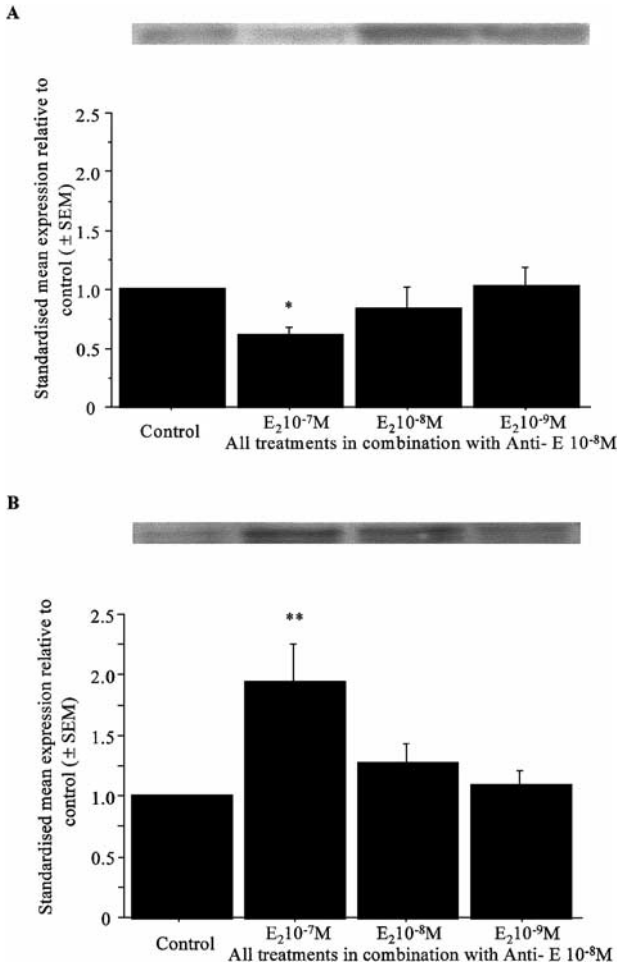

Fig 3. Mean relative protein expression (± SE) of E_2 treatments from 10^{-9} mol/L (M) to 10^{-7} mol/L in combination with anti-E 10^{-8} mol/L compared to control for (A) LPL (n = 5) and (B) HSL (n = 5) in isolated mature SC adipocytes (*P < .05, **P < .01). A representative Western blot is shown for both of the graphs.

central obesity, which may be limited or reversed by HRT.[6,7,29] Our findings provide the first in vitro evidence that E_2 has a regulatory effect on key enzymes of lipogenesis and lipolysis in SC abdominal adipose tissue from women. These results suggest that E_2 may regulate adipose tissue mass by influencing the net amount of adipose tissue present in the adipocyte. Taken with previous data showing E_2 increases the proliferation[15,16] and differentiation[16] of preadipocytes, this indicates that E_2 has a role in the regulation of fat mass.

Our results obtained following treatment with E_2 10^{-7} mol/L showed a statistically significant reduction in LPL expression and an increase in both HSL expression and activity relative to control in keeping with work in animals.[17-21] Furthermore, our results agree with work in human gluteal adipose tissue showing that E_2 reduces lipogenesis.[22,23] In vivo work using transdermal E_2, for 3 weeks at a distant site prior to adipose tissue biopsy, did not have a significant effect on LPL activity or lipolysis in sc abdominal adipose tissue.[24] No information about the concentration of E_2 in the various adipose tissue depots following transdermal therapy is available. Lower levels at a distant adipose tissue depot than in an underlying adipose

Table 1. Mean Glycerol Release Into Medium Following E_2

Treatment	Glycerol (μmol/L per 500,000 cells)
Control	353.2 ± 17.7
$E_2 10^{-12}$ mol/L	452.5 ± 26.7 ↑ *
$E_2 10^{-11}$ mol/L	453.4 ± 24.7 ↑ *
$E_2 10^{-10}$ mol/L	469.6 ± 27.7 ↑ *
$E_2 10^{-9}$ mol/L	471.6 ± 25.0 ↑ *
$E_2 10^{-8}$ mol/L	471.5 ± 33.5 ↑ *
$E_2 10^{-7}$ mol/L	477.7 ± 30.3 ↑ *

NOTE. Total mean glycerol release from isolated mature SC adipocytes into the medium after 48 hours following $E_2 10^{-12}$mol/L to $E_2 10^{-7}$mol/L for n = 10. Analysis repeated in triplicate for each sample. Values given as mean ± SE.

*P < .01.

Table 2. Mean Glycerol Release Into Medium Following Anti-E 10^{-9} mol/L to 10^{-7} mol/L and E_2 10^{-9} mol/L to 10^{-7} mol/L in Combination With Anti-E 10^{-8} mol/L

Treatment	Glycerol (μmol/L per 500,000 cells)
Control	316.6 ± 20.7
Anti-E 10^{-9} mol/L	343.9 ± 44.6
Anti-E 10^{-8} mol/L	366.0 ± 63.2
Anti-E 10^{-7} mol/L	348.7 ± 35.1
$E_2 10^{-9}$mol/L/anti-E 10^{-8} mol/L	347.1 ± 44.8
$E_2 10^{-8}$mol/L/anti-E 10^{-8} mol/L	319.9 ± 20.3
$E_2 10^{-7}$mol/L/anti-E 10^{-8} mol/L	433.8 ± 41.9 ↑ *

NOTE. Total mean glycerol release into medium from isolated mature SC adipocytes after 48 hours following anti-E 10^{-9} mol/L to anti-E 10^{-7} mol/L and $E_2 10^{-9}$ mol/L to $E_2 10^{-7}$ mol/L with anti-E 10^{-8} mol/L for n = 8. Analysis repeated in triplicate for each sample. Values given as mean ± SE.

*$P < .01$.

tissue depot could explain the observed lack of effect since adipose tissue biopsies underlying a transdermal E_2 patch in the gluteal region have been shown to have reduced LPL activity.[23] Our work does, however, suggest a possible dual action of E_2, depending on the concentration of E_2, with the lower concentrations of E_2 increasing LPL expression relative to control.

The reduction in lipogenesis and increase in lipolysis suggested by our work at E_2 10^{-7} mol/L supports the recent review about the effects that HRT has on weight.[30] This work concluded that HRT, either as estrogen alone or in combination with progestogen, is not associated with weight gain. Weight gain is a particular concern among many women taking or considering taking HRT.

The effects of E_2 that we demonstrated differ from those following biopsy of SC abdominal adipose tissue after oral 50 μg ethinyl estradiol (EE) for 3 weeks, which showed no effect on LPL activity and reduced lipolysis as assessed by glycerol release.[24] This was the first time that the effect of estrogen on lipolysis in humans was recognised. The finding also differs from adipose tissue work in animals using E_2 and 2-fluoroestradiol,[19-21] and from that anticipated when the increase in serum TG seen in women taking oral estrogens is considered.[26] The type of estrogen used could provide an explanation since the relative concentrations of estrogen in fat following in vivo administration are not known. Synthetic estrogens do, however, have notably higher systemic potencies than the naturally occurring estrogens.[31] Furthermore, oral EE has been shown to increase serum TG by a greater degree than other oral estrogens.[32]

The discovery of the ER and its subtypes (ERα and β) in human adipose tissue[33,34] provides a potential mechanism by which estrogen exerts its effects in human adipose tissue. In man, a mutation of the ER gene was associated with estrogen resistance; this was manifest clinically as obesity, tall stature, incomplete epiphyseal closure, genu valgum, osteoporosis, acanthosis nigricans, impaired glucose tolerance, and insulin resistance.[35] Work using ER knock-out mice has allowed investigation of the mechanism of action of estrogen in adipose tissue, with the effects appearing to be mediated via the ER.[36,37] The influence of anti-E on lipogenesis and lipolysis observed in our work suggests that the specific in vitro effects of E_2 on lipogenesis and lipolysis occur, at least in part, via the ER. Animal work has shown ICI:compound 182,780 to block nuclear binding to ER in rat adipose tissue, with modulation of the effects of E_2 on adipose tissue seen when given in combination.[38] The anti-estrogen that we used, ICI:compound 182,780, was originally thought to be a "pure" anti-estrogen.[39] Recent work suggests that in addition to anti-estrogenic properties, this so-called "pure" anti-estrogen, ICI:compound 182,780 exhibits estrogenic activity.[40] Our in vitro work suggests that anti-E possesses intrinsic agonist activity as well as antagonist properties in human sc adipose tissue. Further investigation of the effects of both E_2 and anti-E in other adipose tissue depots may provide an insight into the potential for manipulating body fat distribution.

In summary, we have demonstrated for the first time that, in sc abdominal adipocytes from women, E_2 10^{-7} mol/L reduces LPL protein expression, while increasing both HSL protein expression and lipolytic activity in vitro. Furthermore, the effects of anti-E on LPL and HSL protein expression suggest that as in other target organs, the effects of E_2 on human adipose tissue are, at least partly, mediated via the ER. These findings provide an explanation for the observed changes in the lipid profile and fat distribution of postmenopausal women treated with HRT. Further work using E_2, anti-E, and the recently developed selective estrogen receptor modulators (SERMs) may provide novel ways to regulate body fat in postmenopausal women and potentially even influence morbidity associated with central obesity.

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