17 β -Estradiol inhibits 11 β -hydroxysteroid dehydrogenase type 1 activity in rodent adipocytes

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Abstract

17 β -Estradiol (E₂) serves as an anti-obesity steroid; however, the mechanism underlying this effect has not been fully clarified. The effect of E₂ on adipocytes opposes that of glucocorticoids, which potentiate adipogenesis and anabolic lipid metabolism. The key to the intracellular activation of glucocorticoid in adipocytes is 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1), which catalyses the production of active glucocorticoids (cortisol in humans and corticosterone in rodents) from inactive 11-keto steroids (cortisone in humans and 11-dehydrocorticosterone in rodents). Using differentiated 3T3-L1 adipocytes, we showed that E₂ inhibited 11 β -HSD1 activity. Estrogen receptor (ER) antagonists, ICI-182 780 and tamoxifen, failed to reverse this inhibition. A significant inhibitory effect of E₂ on 11 β -HSD1 activity was

observed within 5–10 min. Furthermore, acetylation or α -epimerization of 17-hydroxy group of E₂ attenuated the inhibitory effect on 11 β -HSD1. These results indicate that the inhibition of 11 β -HSD1 by E₂ depends on neither an ER-dependent route, transcriptional pathway nor non-specific fashion. Hexose-6-phosphate dehydrogenase, which provides the cofactor NADPH for full activation of 11 β -HSD1, was unaffected by E₂. A kinetic study revealed that E₂ acted as a non-competitive inhibitor of 11 β -HSD1. The inhibitory effect of E₂ on 11 β -HSD1 was reproduced in adipocytes isolated from rat mesenteric fat depots. This is the first demonstration that E₂ inhibits 11 β -HSD1, thereby providing a novel insight into the anti-obesity mechanism of estrogen.

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Introduction

Rodents that undergo an ovariectomy become obese and 17 β -estradiol (E₂) prevents the progression of this obesity (Pedersen *et al.* 1991, 1992). In humans, fat accumulation occurs in postmenopausal women suggesting an inhibitory role of estrogen in fat deposition (Haarbo *et al.* 1991, Stampfer *et al.* 1991). Glucocorticoids also play a pivotal role in regulating fat metabolism and distribution (Kahn & Flier 2000, Montague & O'Rahilly 2000, Wajchenberg 2000, Masuzaki *et al.* 2003). Patients suffering from hypercortisolemia due to Cushing's syndrome and those undergoing corticosteroid therapy demonstrate an increase in body fat mass (Rebuffe-Scrive, *et al.* 1988, Mayo-Smith *et al.* 1989). However, circulating glucocorticoid concentrations are not elevated in most obese or type 2 diabetes subjects (Chrousos & Gold 1992, Hautanen *et al.* 1997, Phillips *et al.* 1998, Bjorntorp & Rosmond 2000). The key enzyme in intracellular glucocorticoid activation is 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1), which generates active glucocorticoids (cortisol in humans and corticosterone in rodents) from inactive metabolites (cortisone in humans and

11-dehydrocorticosterone in rodents; Stewart & Krozowski 1999). A cofactor, NADPH, supplied by hexose-6-phosphate dehydrogenase (H6PD) in the endoplasmic reticulum, is critical for full activation of 11 β -HSD1 (Hewitt *et al.* 2005, McCormick *et al.* 2006, Marcolongo *et al.* 2007).

The importance of local activation of glucocorticoids in adipose tissue has been demonstrated in transgenic mice overexpressing 11 β -HSD1 under the control of the adipose-specific aP2 promoter/enhancer (Masuzaki *et al.* 2001). Transgenics express ~3-fold higher 11 β -HSD1 levels than normal mice and develop full metabolic syndrome, characterized by visceral obesity, insulin-resistant diabetes, hyperlipidemia, and high arterial blood pressure (Masuzaki *et al.* 2001, 2003). By contrast, 11 β -HSD1 knockout mice exhibit an apparently insulin-sensitive phenotype and resist visceral fat accumulation, even when fed a chronic high-fat diet (Kotelevtsev *et al.* 1997, Morton *et al.* 2001). Similarly, the suppression of 11 β -HSD1 activity by pharmacological inhibitors, such as arylsulfonamidothiazoles and carbenoxolone, results in beneficial effects on the liver and adipose tissue. Arylsulfonamidothiazoles are effective for enhancing hepatic insulin sensitivity and lowering blood glucose levels

in diabetic mice (Alberts *et al.* 2002, 2003). In humans, carbenoxolone has been shown to enhance hepatic insulin sensitivity in healthy men and in type 2 diabetic patients (Walker *et al.* 1995, Andrews *et al.* 2003). Therefore, the suppression of 11 β -HSD1 activity is of great significance for the potential treatment of obesity and metabolic syndrome.

These findings prompted us to investigate the effect of E₂ on 11 β -HSD1 activity. In the present study, we demonstrated that E₂ acted as a non-competitive inhibitor of 11 β -HSD1 and inhibition occurred on an estrogen receptor (ER)-independent and non-transcriptional pathway in rodent adipocytes.

Materials and Methods

Animals

Male Wistar rats (7-week-old) were obtained from SLC Co. Ltd (Shizuoka, Japan). They were housed under a controlled 12 h light:12 h darkness cycle (light from 0700 to 1900 h) with room temperature at 23 $\pm$ 1 °C and humidity at 55 $\pm$ 5%, and had access to food *ad libitum*. After a resting period of 1 week, the animals were killed by bleeding from the abdominal aorta under light ether anesthesia. The experimental procedures were approved by the Kobe Pharmaceutical University Animal Care and Use Committee.

Culture and differentiation of 3T3-L1 cells

The 3T3-L1 preadipocyte cell line was cultured in DMEM containing 10% calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were plated at a density of 4 $\times$ 10⁴ cells per ml and maintained at 37 °C in a humidified atmosphere of 10% CO₂/90% air. Two-day postconfluent 3T3-L1 cells (designated as day 0) were differentiated into adipocytes in 6- or 12-well plates (Iwaki, Tokyo, Japan) by treatment for 3 days with 2.5 μ M dexamethasone (Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 μ g/ml insulin (Roche), and antibiotics in DMEM supplemented with 10% fetal bovine serum (FBS, GIBCO). Differentiation was completed by incubation with 1 μ g/ml insulin for an additional 3 days in DMEM with 10% FBS and antibiotics. From day 6 onward, cells were fed DMEM containing 10% FBS and antibiotics. More than 95% of cells were transformed into adipocytes, as evaluated by the detection of lipid droplets by phase contrast microscopy and oil-red O staining (data not shown). This population was referred to as '3T3-L1 adipocytes'.

Microsome preparation

Microsomes were prepared from 3T3-L1 adipocytes and mesenteric fat pads of 8-week-old male Wistar rats according to the method described elsewhere with minor modification (Kobayashi *et al.* 1987). Microsomes were reconstituted with 0.07 M phosphate buffer, pH 7.4 containing 0.25 M sucrose.

We confirmed no contamination of the supernatant fraction in the microsomal preparation by enzyme assay for alcohol dehydrogenase, which was localized in the supernatant fraction but not in microsomes, which were rapidly frozen in liquid nitrogen and stored at -80 °C until use. Protein concentration of microsomes was determined using Bio-Rad Protein Assay solution with BSA as the standard, according to the manufacturer's instructions.

Preparation of isolated adipocytes from rats

Isolated adipocytes were prepared from the whole mesenteric fat pads of 8-week-old male Wistar rats by the method of Rodbell (1964). In brief, the whole mesenteric fat depots (10–12 g) of six rats were removed, minced, and digested at 37 °C with 50 mg of type 1 collagenase (Worthington Biochemicals, Lakewood, NJ, USA) in 25 ml DMEM, pH 7.4, containing 2% BSA. Following 45–50 min of digestion, the liberated cells were filtrated through a 150 μ m nylon screen and the filtrate was washed with DMEM containing 2% BSA twice. Then, the filtrate was centrifuged at 600 g for 30 s. The floating layer was used as isolated adipocytes.

Measurement of 11 β -HSD1 activity

11 β -HSD1 activity (11-oxoreductase) was measured using adipocytes from 3T3-L1 a) and mesenteric fat depots of rats b), and microsomes from rat mesenteric fat depots c) and 3T3-L1 adipocytes d).

- (a) 3T3-L1 adipocytes were carefully washed in PBS (–) buffer, and then preincubated in DMEM for 5 min. Immediately before starting the reaction, DMEM was changed to medium containing 1 μ M 11-dehydrocorticosterone (Sigma) with or without E₂ (Sigma). Incubation was carried out in 6- or 12-well plates at 37 °C for 5–10 min in a humidified atmosphere in the presence of 10% CO₂. The final concentration of dimethylsulfoxide (DMSO) solution, which dissolved the steroids, was below 0.05–0.1% in the incubation medium, which was transferred to a tube containing 20 μ l of concentrated phosphoric acid. The medium was added to the solid-phase extraction column (Oasis HLB 30 mg Extraction Cartridges, Waters, Milford, MA, USA), and the column was washed with 3 ml water. Steroids were eluted from the column with 2 ml methanol, and the methanol solution was evaporated *in vacuo*. Then, 400 μ l HPLC mobile-phase solution (H₂O:methanol=45:55, v/v%, solvent A) was added to the tube. The concentration of 11-dehydrocorticosterone and corticosterone in the sample (10 μ l) was measured with an HPLC system (NANOSPACE SI-1, Shiseido Co. Ltd, Tokyo, Japan). The steroid was separated with Capcellpak UG 120, 5 μ m, 250 mm $\times$ 1.5 mm ID column (Shiseido) at 40 °C and detected at 240 nm. The column was eluted with solvent A for 45 min.

The flow rate was 0.1 ml/min. Under these conditions, 11-dehydrocorticosterone and corticosterone appeared at 16.27 ± 0.04 min (mean $\pm$ s.d., $n=5$) and 26.36 ± 0.07 min ($n=5$) respectively. The intra-assay coefficients of variation (CV) for both steroids were $4.1\text{--}5.3\%$ ($n=5$) and the values for the inter-assay CV were $7.8\text{--}9.2\%$ ($n=5$). The recoveries were $94.4 \pm 5.1\%$ ($n=5$) for 11-dehydrocorticosterone and $96.2 \pm 3.9\%$ ($n=5$) for corticosterone. These steroids separated by HPLC were ascertained by gas chromatography/mass spectrometry. To validate the above assay, it was necessary to establish that steroid formation was proportional to both the incubation time and protein concentrations. The appearance of corticosterone was linear for a period of 10 min in the six-well plate and was proportional to the protein concentration (data not shown).

- (b) To measure 11 β -HSD1 activity in isolated adipocytes from mesenteric fat pads, 100 μ l (1.5×10^6 cells/ml) adipocytes was added to DMEM containing 10% charcoal-stripped FBS (Biowest, Miami, FL, USA) and 1 μ M 11-dehydrocorticosterone with or without E_2 . Incubation was carried out in six-well plates at 37 °C for 30 min in a humidified atmosphere in the presence of 10% CO₂. The subsequent procedures were the same as described in the method (a). Under these conditions, the appearance of corticosterone was linear for 30 min (data not shown).
- (c) 11 β -HSD1 activity in microsomes from rat mesenteric fat depots was determined in MOPS buffer (100 mM KCl, 20 mM NaCl, and 20 mM MOPS, pH 7.4) containing 1 mM NADPH (Oriental Yeast, Tokyo, Japan), and 11-dehydrocorticosterone (1 μ M) with and without E_2 . The reaction was started by the addition of 10 μ l microsomes (0.15 mg protein/ml) and the reactants were incubated at 37 °C for 30 min. Steroid concentrations were measured by HPLC by the same method shown in (a). Under these conditions, the appearance of corticosterone was linear for 30 min (data not shown).
- (d) 11 β -HSD1 activity in microsomes from 3T3-L1 adipocytes was determined in 1 ml MOPS buffer containing 6 mM glucose 6-phosphate (G-6-P, Oriental Yeast), 1 mM NADP⁺ (Oriental Yeast), 0.35 units/ml glucose-6-phosphate dehydrogenase (G6PD, Oriental Yeast), and 1 μ M 11-dehydrocorticosterone (NADPH-regenerating system). The enzyme reaction was initiated by the addition of microsome (15 μ l, 1.3 mg protein/ml), and the reactants were incubated at 37 °C for 60 min. The reaction was stopped by the addition of 2 ml CH₂Cl₂ solution to the tubes. Extracted steroids were measured by HPLC as above. Linearity of enzyme activity versus time (up to 60 min) and the protein concentration were confirmed (data not shown). To assess dehydrogenase activity (11 β -HSD2 activity), 5 μ M corticosterone (Sigma) was added to the above system in place of 11-dehydrocorticosterone.

Measurement of H6PD activity

H6PD activity was measured by fluorometric detection of NADPH produced in the MOPS buffer (see above) containing 6 mM G-6-P and 1 mM NADP⁺ at 22 °C. The reaction was started by the addition of microsomes (20 μ l, 0.15 mg/ml) to the cuvette. Steroids were dissolved in DMSO and added to the MOPS buffer at a final concentration of DMSO below 0.05%. Changes in fluorescent intensity (340 nm excitation and 460 nm emission wavelengths) were measured using a Shimadzu RF 510 fluorescence spectrophotometer (Shimadzu, Kyoto, Japan). NADPH concentration was measured by a standard curve obtained from NADPH pure chemical.

Kinetic assay

Enzyme reactions were carried out in 1 ml MOPS buffer containing 0.25–1 μ M 11-dehydrocorticosterone, 6 mM G-6-P, 1 mM NADP⁺, and 0.35 units/ml G6PD at 37 °C for 60 min. If necessary, 5–20 μ M E_2 was added to the buffer. The reaction was initiated by the addition of microsome (15 μ l, 1.3 mg/ml). Steroid concentrations were measured by HPLC as above. Linearity of enzyme activity versus time and protein concentration was confirmed. K_m value estimations were averaged from Lineweaver–Burke plots with triplicate samples.

Statistical analysis

Data were expressed as the mean $\pm$ s.e.m. Statistical analysis was done using Dunnett's test or Tukey's test for multiple comparisons. Differences between two groups were statistically compared by the two sample *t*-test. *P* values below 0.05 were considered significant.

Results

Effect of E_2 on 11 β -HSD1 activity in 3T3-L1 adipocytes

To investigate the effect of E_2 on 11 β -HSD1 activity, differentiated 3T3-L1 adipocytes were incubated in DMEM containing 11-dehydrocorticosterone with and without E_2 . The suppression of 11 β -HSD1 activity by E_2 (5–25 μ M) occurred in a dose-dependent manner (Fig. 1A). Moreover, the inhibition of E_2 was observed within 5 min (Fig. 1B). In these 3T3-L1 adipocytes, we were not able to measure any conversion of corticosterone to 11-dehydrocorticosterone even after extended (8 h) incubation with the substrate, indicating that 11 β -HSD1 functions almost exclusively as a reductase in differentiated 3T3-L1 adipocytes and that 11 β -HSD2 activity is absent from these cells (data not shown).

Figure 1 Inhibition of 11 β -HSD1 activity by 17 β -estradiol in differentiated 3T3-L1 adipocytes. 3T3-L1 cells were grown to confluence and differentiated into adipocytes. Adipocytes were incubated in DMEM in a humidified atmosphere in the presence of 10% CO₂; (A) with or without varying concentrations of 17 β -estradiol (1–25 μ M) together with 11-dehydrocorticosterone (1 μ M) at 37 °C for 10 min ($n=5$); (B) with or without 17 β -estradiol (50 μ M) together with 11-dehydrocorticosterone at 37 °C for 5 and 10 min ($n=3$). Corticosterone levels in the medium were determined by HPLC. Data are the mean $\pm$ S.E.M. (A) *, $P<0.05$; **, $P<0.01$ versus vehicle-treated cells (Dunnett's test). (B) *, $P<0.05$; **, $P<0.01$ (two sample t -test).

Specificity of the inhibitory effect of E₂ on 11 β -HSD1 activity

To test specificity of the inhibitory effect of E₂ on 11 β -HSD1 activity, 3T3-L1 adipocytes were incubated in DMEM containing 11-dehydrocorticosterone with 50 μ M of 17 α -E₂, E₂ 3-acetate (E₂-3-AcO), E₂ 17-acetate (E₂-17-AcO), or E₂ 3, 17-diacetate (E₂-3,17-diAcO). Among these estradiol derivatives tested, E₂ and E₂-3-AcO inhibited 11 β -HSD1 most potently (Fig. 2). Acetylation or α -epimerization of 17-hydroxy group of E₂ attenuated the inhibitory effect on 11 β -HSD1. These findings suggest that 17-hydroxy group of E₂ is significant for the inhibitory effect of 11 β -HSD1 activity and the inhibition by E₂ is specific for 11 β -HSD1.

ER blockers do not influence the inhibitory effect of E₂ on 11 β -HSD1 activity

Steroid receptors have traditionally been thought of as nuclear receptors, which exert their function as transcription factors. Recently, it has been accepted that ER α exists and functions as a non-traditional G-protein-coupled receptor at the plasma membrane. It has been suggested that such membrane receptors mediate rapid non-genomic steroid-signaling events (Falkenstein *et al.* 2000, Hall *et al.* 2001, Thomas *et al.* 2005). Thus, we next investigated the involvement of ERs in the 11 β -HSD1 inhibitory effect of E₂ on 3T3-L1 adipocytes by using ER antagonists, ICI-182 780 (ICI, Tocris Bioscience, St Louis, MO, USA) and tamoxifen (TAM, Wako Pure Chemical Industry, Osaka, Japan). 3T3-L1 adipocytes were incubated in DMEM containing E₂ and 11-dehydrocorticosterone with or without ICI (1 μ M). 3T3-L1 adipocytes were also incubated in DMEM containing TAM (10 μ M) prior to the addition of E₂ for 1 h. Then E₂ and 11-dehydrocorticosterone were added to six-well plates and incubated at 37 °C for 10 min. A 60% inhibition was observed by E₂ (50 μ M) and this inhibition was not recovered in the presence of ICI or TAM (Fig. 3).

E₂ inhibits 11 β -HSD1 activity in rat adipocytes

To explore the physiological relevance of the above findings, we next investigated the inhibitory effect of E₂ on adipocytes obtained from mesenteric fat depots of Wistar rats. The adipocytes were incubated in DMEM containing 10% charcoal-stripped FBS and 11-dehydrocorticosterone with and without E₂ for 30 min. The suppression of 11 β -HSD1 activity by E₂ (15–25 μ M) occurred in a dose-dependent manner (Fig. 4).

Figure 2 Specificity of the inhibitory effect of 17 β -estradiol on 11 β -HSD1 activity. 3T3-L1 adipocytes were incubated in DMEM containing 1 μ M 11-dehydrocorticosterone with 50 μ M of 17 α -estradiol (17 α -E₂), 17 β -estradiol 3-acetate (E₂-3-AcO), 17 β -estradiol 17-acetate (E₂-17-AcO), or 17 β -estradiol 3, 17-diacetate (E₂-3,17-diAcO) in a humidified atmosphere in the presence of 10% CO₂ at 37 °C for 10 min. Data are the mean $\pm$ S.E.M. ($n=3$). *, $P<0.05$; **, $P<0.01$ versus vehicle-treated cells (Tukey's test). NS, not significant.

Figure 3 Effect of TAM and ICI on inhibition of 11 β -HSD1 activity by 17 β -estradiol. 3T3-L1 adipocytes were incubated in DMEM containing 17 β -estradiol (50 μ M) and 11-dehydrocorticosterone in a humidified atmosphere in the presence of 10% CO₂ with and without ICI (1 μ M) in six-well plates at 37 °C for 10 min. Adipocytes were also incubated in DMEM containing TAM (10 μ M) prior to the addition of steroids for 1 h. Then, 17 β -estradiol and 11-dehydrocorticosterone were added and incubated at 37 °C for 10 min. Corticosterone levels in the medium were determined by HPLC. Data are the mean $\pm$ s.e.m. ($n=3$). *, $P<0.05$; **, $P<0.01$ versus each control (zero concentration of estradiol; slant bar, + estradiol; gray bar, +10 μ M TAM; black bar, 1 μ M ICI; two sample t -test).

E₂ inhibits 11 β -HSD1 in microsomes from rat mesenteric fat pads

We next determined whether E₂ inhibited 11 β -HSD1 in a cell-free system by using microsomes from rat mesenteric fat pads with or without E₂. As shown in Fig. 5A, significant inhibition of 11 β -HSD1 activity was observed by

Figure 4 Inhibitory effect of 17 β -estradiol on 11 β -HSD1 activity in adipocytes from rat mesenteric fat depots. Adipocytes obtained from mesenteric fat depots of Wistar rats were incubated in DMEM containing 10% charcoal-stripped FBS and 11-dehydrocorticosterone with and without 17 β -estradiol in six-well plates at 37 °C for 30 min in a humidified atmosphere in the presence of 10% CO₂. Corticosterone levels in the medium were determined by HPLC. Data are the mean $\pm$ s.e.m. ($n=5$). *, $P<0.05$; **, $P<0.01$ versus vehicle-treated cells (Dunnett's test).

Figure 5 Effect of 17 β -estradiol on 11 β -HSD1 and H6PD activity in microsomes from rat mesenteric fat depots. (A) 11 β -HSD1 activity was determined in MOPS buffer containing NADPH (1 mM) and 11-dehydrocorticosterone (1 μ M) with and without 17 β -estradiol. The reaction was started by the addition of 10 μ l microsomes (0.15 mg protein/ml), and the reactants were incubated at 37 °C for 30 min. Steroid concentrations were measured by HPLC. Data are the mean $\pm$ s.e.m. ($n=5$). *, $P<0.05$; **, $P<0.01$ versus vehicle-treated cells (Dunnett's test). (B) H6PD activity was measured by fluorometric detection of NADPH in MOPS buffer containing G-6-P (6 mM) and NADP⁺ (1 mM) with or without 17 β -estradiol. The reaction was started by the addition of microsomes (20 μ l, 0.15 mg protein/ml) to the cuvette ($n=3$). Changes in the fluorescence intensity were measured at 340 nm excitation and 460 nm emission wavelengths for 30 min at 22 °C. NADPH concentration was measured by a standard curve obtained from pure NADPH. Closed circles, 0 μ M estradiol (E₂); open circles, 25 μ M E₂; closed triangles, 50 μ M E₂; open triangles, microsomes heated.

the addition of E₂ (0.5–1 μ M) in a dose-dependent manner. Under these incubation conditions, only about 20% of dehydrogenase activity (conversion of corticosterone to 11-dehydrocorticosterone) compared with 11 β -HSD1 activity was observed (data not shown).

Lack of inhibition of H6PD activity and G-6-P transporter by E₂ in microsomes from rat mesenteric fat pads

H6PD and 11 β -HSD1 are both located in the intralumen of the endoplasmic reticulum (Hewitt *et al.* 2005). The coupling of activities of H6PD and 11 β -HSD1 has been suggested in early studies on the basis of cofactor requirements, i.e. H6PD

uses G-6-P and NADP⁺, and generates NADPH, which is a cofactor for 11-oxoreduction reaction of 11 β -HSD1 (Sawada *et al.* 1980, 1981). Recently, McCormick *et al.* (2006) have also reported the presence of an interconnection between the pentose pathway and 11 β -HSD1 activity in isolated rat adipocytes and rat liver microsomes. Therefore, we determined the effects of E₂ on H6PD in intact microsomes. The integrity of the microsomal membrane was inferred from the latency of glucose dehydrogenase activity (Bublitz & Steavenson 1988b, Romanelli *et al.* 1994), which was found to be >93% in all the preparations assayed. The reaction was started by the addition of microsomes, as described in the Materials and Methods section. In the absence of E₂, a linear increase in fluorescence intensity due to the generation of NADPH (H6PD activity) was observed up to 30 min, and E₂ (25–50 μ M) failed to inhibit this increase in NADPH production (Fig. 5B). These results indicate that the inhibitory effect of E₂ on 11 β -HSD1 activity may not be mediated through the decrease in NADPH generation by the suppression of H6PD in the intraluminal compartment of the endoplasmic reticulum (Beutler & Morrison 1967, Bublitz & Steavenson 1988a). In addition, these data also show that E₂ does not inhibit G-6-P transporter (G-6-P-T), which is located in the membrane of the endoplasmic reticulum and supplies G-6-P into the lumen (Chou *et al.* 2002).

Kinetic study of microsomal 11 β -HSD1 in 3T3-L1 adipocytes

To substantiate the above inhibitory effect of E₂ on 11 β -HSD1, we next conducted kinetic studies to determine how E₂ regulates enzyme activities. The action of E₂ on 11 β -HSD1 of 3T3-L1 adipocyte microsomes was investigated, and the results plotted according to Lineweaver & Burk (1934; Fig. 6). These results indicated that E₂ acted as a non-competitive inhibitor of 11 β -HSD1, as the slopes of the four curves (without E₂ and with three different levels of E₂) were different, but all converged to the same point of one per substrate axis (x -axis). The apparent K_m value for 11-dehydrocorticosterone was 0.25 μ M, which is consistent with the values of 0.12 and 0.25 μ M obtained by Kotelevtsev *et al.* (1997) and McCormick *et al.* (2006) respectively. These results suggest that E₂ might exert its inhibitory effect by interacting with the enzyme 11 β -HSD1 in 3T3-L1 adipocyte microsomes.

Discussion

The major finding of the present study was that, in rodent adipocytes, E₂ inhibited 11 β -HSD1 activity in an ER-independent and non-transcriptional pathway. We showed here for the first time that E₂ interacted with 11 β -HSD1 as a non-competitive inhibitor. It has been reported that E₂ stimulates 11 β -HSD1 mRNA expression in preadipocytes from women, but not in those from men (Dieudonne *et al.* 2006). More recently, it has been demonstrated that no effect

Figure 6 Kinetic study (Lineweaver–Burke plots). Enzyme reactions were carried out in 1 ml MOPS buffer containing 11-dehydrocorticosterone (0.25–1 μ M), G-6-P (6 mM), NADP⁺ (1 mM), and G6PD (0.35 units/ml) with and without 17 β -estradiol (E₂, 5–20 μ M) at 37 °C for 60 min. The reaction was initiated by the addition of 3T3-L1 adipocyte microsomes (15 μ l, 1.3 mg/ml). Steroid concentrations were measured by HPLC. Data are the mean $\pm$ S.E.M. ($n=3$).

of E₂ on 11 β -HSD1 mRNA expression is observed in adipose tissue from healthy women (Paulsen *et al.* 2008). In the present study, E₂ did not reduce 11 β -HSD1 mRNA levels in 3T3-L1 adipocytes under the assay conditions (data not shown). Furthermore, 11 β -HSD1 inhibition was observed within 5 min (Fig. 1B). Thus, it is unlikely that E₂ inhibited 11 β -HSD1 by the reduction of 11 β -HSD1 mRNA expression in the present study. It has been reported that non-genomic biological effects of E₂ are mediated through membrane-bound subpopulations of ER α and β (Pappas *et al.* 1995, Razandi *et al.* 1999, Evinger & Levin 2005, Revankar *et al.* 2005, Thomas *et al.* 2005). However, in the present study, ER blockers, TAM and ICI, did not reverse the inhibitory effect of E₂ on 11 β -HSD1 (Fig. 3). Furthermore, acetylation or α -epimerization of 17-hydroxy group of E₂ attenuated the inhibitory effect on 11 β -HSD1 (Fig. 2). Taken together, our data suggest that the suppressive effect of E₂ on 11 β -HSD1 in adipocytes may not depend on the transcriptional pathway, ER-dependent route, or non-specific fashion.

H6PD supplies NADPH, a critical cofactor for the full activation of 11 β -HSD1 (Atanasov *et al.* 2004, McCormick *et al.* 2006, Marcolongo *et al.* 2007). Consistent with this fact, H6PD knockout mice lack 11 β -HSD1 activity (Lavery *et al.* 2006). In this context, we investigated the effect of E₂ on H6PD activity, and our results demonstrated that E₂ did not inhibit H6PD in intact microsomes from rat mesenteric fat depots (Fig. 5B). As H6PD activity was unchanged by E₂, it is unlikely that the inhibitory effect of E₂ occurred through suppression of the G-6-P-T, which is located in the

membrane of the endoplasmic reticulum and transports the substrate of H6PD, G-6-P, into the lumen (Chou *et al.* 2002, Marcolongo *et al.* 2007).

The present study showed that the inhibition of 11 β -HSD1 by E₂ occurred not only in 3T3-L1 adipocytes, but also in adipocytes obtained from mesenteric fat depots in rats (Fig. 4). This suggests that the inhibitory effect of E₂ on 11 β -HSD1 can be generalized in rodent adipocytes.

It is widely accepted that most of the extra-gonadal conversion of androgens into estrogens by aromatase occurs in stromal-vascular cells of adipose tissue (Ackerman *et al.* 1981). Estrogens produced within this compartment are biologically active only at the local-tissue level in a paracrine or intracrine fashion (Labrie *et al.* 1997). Moreover, estrogen levels in this compartment may be at least an order of magnitude greater than those in the circulating plasma (Rodriguez-Cuenca *et al.* 2005). In agreement with this observation, the total steroid content of adipose tissue (estimated with a mean body fat mass of 20 kg) is 40–400 times greater than the total steroid content of plasma (assuming a 3 L plasma volume; Deslypere *et al.* 1985). Furthermore, stromal-vascular cells in adipose tissue may be considered as an estrogen-producing microdomain. Interestingly, an example for the microdomain hypothesis can be seen in 11 β -HSD1 enzyme system. 11 β -HSD1, which resides in the lumen of the endoplasmic reticulum membrane, has a low affinity for its substrate. *K_m* values in the present data and by the others (Kotelevtsev *et al.* 1997, Maser *et al.* 2002, McCormick *et al.* 2006), ranging from low micromolar to submicromolar values, are higher than the nanomolar-circulating substrate concentration (Nomura *et al.* 1997, Tagawa *et al.* 2007). One explanation for this paradox is the proximity of the enzyme to the endoplasmic reticulum membrane (Lakshmi & Monder 1988, Walker *et al.* 2001, Hosfield *et al.* 2005, Zhang *et al.* 2005). In the membrane, a potentially higher membrane-localized substrate concentration by metabolism can drive the catalysis even though the enzyme has low affinity for its substrate. It is therefore not surprising that 11 β -HSD1 activity was inhibited by relatively high doses of E₂ *in vitro* in rodent adipocytes.

The importance of 11 β -HSD1-mediated local amplification of glucocorticoids in the development of visceral obesity and its associated metabolic disturbances is being increasingly recognized (Livingstone *et al.* 2000, Masuzaki *et al.* 2001, Paulmyer-Lacroix *et al.* 2002), and is supported by the development of visceral obesity and metabolic syndrome in mice overexpressing white adipose 11 β -HSD1 (Masuzaki *et al.* 2001). Furthermore, it has been reported that 11 β -HSD1 mRNA expression is increased in both visceral and s.c. adipose tissue of obese patients (Desbriere *et al.* 2006, Paulsen *et al.* 2007). Therefore, 11 β -HSD1 is considered a promising drug target for the treatment of glucocorticoid-dependent metabolic diseases. In fact, pharmacologic inhibition of 11 β -HSD1 can ameliorate multiple facets of metabolic syndrome in mice (Barf *et al.* 2002, Alberts *et al.* 2003, Hermanowski-Vosatka *et al.* 2005). Our findings

suggest that E₂ may exert its effect on 11 β -HSD1 as an endogenous inhibitor in rodents.

Adipose tissue is composed of mature adipocytes and stromal-vascular cells, with estrogen production occurring predominantly in stromal-vascular cells (Ackerman *et al.* 1981, Price *et al.* 1992), and 11 β -HSD1 mRNA expression and enzyme activity occurring predominantly in adipocytes (Berger *et al.* 2001, Atanasov *et al.* 2004, Dieudonne *et al.* 2006). Glucocorticoids activate aromatase activity in cultured stromal-vascular cells of adipose tissue (Simpson *et al.* 1981, Folkerd & James 1983, Perel *et al.* 1986, Dieudonne *et al.* 2006). We showed here that E₂ inhibited 11 β -HSD1 in rodent adipocytes. These findings suggest the possibility of feedback regulation between estrogens from stromal-vascular cells and glucocorticoids from adipocytes. In obese men, the peripheral conversions of testosterone to estradiol and androstenedione to estrone (aromatase activities) are increased in proportion to the degree of obesity (Schneider *et al.* 1979, Tchernof *et al.* 1995). As mentioned above, 11 β -HSD1 mRNA in human adipose tissue is higher in obese subjects than in lean subjects in both women and men (Desbriere *et al.* 2006, Paulsen *et al.* 2007). Although it is not easy to extrapolate human pathophysiology from animal studies, disruption of such feedback may be relevant, at least in part, to dysfunction of adipose tissue in humans.

In conclusion, we demonstrated, for the first time, a non-competitive inhibitory effect of E₂ on 11 β -HSD1 in adipocytes, suggesting a novel method of regulating glucocorticoid metabolism by estrogens. Based on our results, we hypothesize that the anti-obesity effects of E₂ (Homma *et al.* 2000, Jones *et al.* 2000) are attributed, at least in part, to the inhibition of 11 β -HSD1 activity in adipocytes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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