Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11 β -hydroxysteroid dehydrogenase type 1

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Abstract

Two isozymes of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) interconvert active cortisol and inactive cortisone. 11 β -HSD2 (renal) acts only as a dehydrogenase, converting cortisol to cortisone. 11 β -HSD1 (liver) is a bi-directional enzyme in cell homogenates, whereas in intact cells it typically displays oxo-reductase activity, generating cortisol from cortisone. We recently established that cortisone reductase deficiency is a digenic disease requiring mutations in both the gene encoding 11 β -HSD1 and in the gene for a novel enzyme located within the lumen of the endoplasmic reticulum (ER), hexose-6-phosphate dehydrogenase (H6PDH). This latter enzyme generates NADPH, the co-factor required for oxo-reductase activity. Therefore, we hypothesized that H6PDH expression may be an important determinant of 11 β -HSD1 oxo-reductase activity. Transient transfection of chinese hamster ovary (CHO) cells with 11 β -HSD1 resulted in the appearance of both oxo-reductase and dehydrogenase activities in intact cells. Co-transfection of 11 β -HSD1 with H6PDH increased oxo-reductase activity whilst virtually eliminating dehydrogenase activity. In contrast, H6PDH had no effect on reaction direction of 11 β -HSD2, nor did the cytosolic enzyme, glucose-6-phosphate dehydrogenase (G6PD) affect 11 β -HSD1 oxo-reductase activity. Conversely in HEK 293 cells stably transfected with 11 β -HSD1 cDNA, transfection of an H6PDH siRNA reduced 11 β -HSD1 oxo-reductase activity whilst simultaneously increasing 11 β -HSD1 dehydrogenase activity. In human omental preadipocytes obtained from 15 females of variable body mass index (BMI), H6PDH mRNA levels positively correlated with 11 β -HSD1 oxo-reductase activity, independent of 11 β -HSD1 mRNA levels. H6PDH expression increased 5.3-fold across adipocyte differentiation ($P < 0.05$) and was associated with a switch from 11 β -HSD1 dehydrogenase to oxo-reductase activity. In conclusion, H6PDH is a crucial determinant of 11 β -HSD1 oxo-reductase activity in intact cells. Through its interaction with 11 β -HSD1, H6PDH may represent a novel target in the pathogenesis and treatment of obesity.

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Introduction

The enzyme 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1, EC 1.1.1.146) inter-converts active cortisol and inactive cortisone, and therefore represents an important regulatory mechanism for tissue specific glucocorticoid receptor (GR) activation. 11 β -HSD1 resides within the lumen of the endoplasmic reticulum (ER) (Ozols 1995, Odermatt *et al.* 1999) and is bi-directional, being able to catalyze both the dehydrogenase (cortisol to cortisone) and oxo-reductase (cortisone to cortisol) reactions (Agarwal *et al.* 1989). *In vivo* and in intact cells, oxo-reductase activity predominates (Bujalska *et al.* 1997a, Jamieson *et al.* 2000, Masuzaki *et al.* 2001), but in cellular homogenates or in purified preparations, the enzyme behaves as a dehydrogenase (Lakshmi & Monder 1988); oxo-reductase activity can only be recovered in these situations upon the addition

of an artificial NADPH re-generation system, such as can be provided by the activity of glucose-6-phosphate dehydrogenase (G6PD) (Agarwal *et al.* 1990, Walker *et al.* 2001).

11 β -HSD1-mediated oxo-reductase activity in liver and adipose tissue may modulate development of the metabolic syndrome. Exposure to cortisol increases hepatic glucose output (Jones *et al.* 1993) and adipocyte differentiation (Hauner *et al.* 1987). Selective inhibitors have shown beneficial results in improving insulin sensitivity and causing weight loss (Alberts *et al.* 2003, Livingstone & Walker 2003), but the factors determining oxo-reductase activity *per se* have remained elusive.

Hexose-6-phosphate dehydrogenase (H6PDH, EC 1.1.1.47) performs the first two steps of a pentose phosphate pathway which is believed to exist within the ER lumen (Bublitz & Steavenson 1988). It is responsible for converting glucose-6-phosphate to

6-phospho-gluconolactone, thereby generating NADPH (Kimura *et al.* 1979, Stegeman & Klotz 1979). Based on genetic evidence, we have argued that reduced H6PDH activity, together with reduced expression of 11 β -HSD1, underlies cortisone reductase deficiency (CRD). Women affected with CRD have polycystic ovary syndrome (PCOS) with menstrual irregularity, hirsutism and infertility. They are unable to generate cortisol from cortisone due to defective 11 β -HSD1 oxo-reductase activity (Nikkila *et al.* 1993, Jamieson *et al.* 1999) resulting in activation of the hypothalamic–pituitary–adrenal axis and adrenocorticotrophin (ACTH) driven adrenal androgen excess (Biaison-Laubert *et al.* 2000, Laing *et al.* 2002). These patients carry coding-sequence mutations in the gene encoding H6PDH (*H6PD*) in association with intronic mutations in the 11 β -HSD1 gene (*HSD11B*) that reduce gene transcription (Draper *et al.* 2003).

Here we show that H6PDH has the potential to profoundly influence 11 β -HSD1 by affecting the reaction direction in intact cells and also demonstrate that in primary cultures of human omental preadipocytes, levels of H6PDH expression predict oxo-reductase activity.

Materials and methods

Patients

Omental adipose tissue biopsies were obtained from 15 female patients undergoing elective abdominal surgery (median age 40 years, range 28–60 years, median body mass index (BMI) 29.3 kg/m², range 19.7–39.2). All gave their full informed, written consent. The study had the approval of the local research ethics committee.

Primary culture

Omental preadipocytes were isolated as previously reported (Bujalska *et al.* 1997b), seeded in a 24-well tissue culture dish and cultured to confluence (up to 6 days) in DMEM/F12 media supplemented with 10% fetal calf serum. At this point, 11 β -HSD1 oxo-reductase assays were performed, protein concentration measured and total RNA extracted. In a further set of experiments, omental preadipocytes were isolated from adipose tissue obtained at elective surgery ($n=12$). Preadipocytes were differentiated into mature adipocytes according to an established protocol (Hauner *et al.* 1987). Briefly, after cell washing (day 1), 11 β -HSD1 oxo-reductase and dehydrogenase activities were measured and total RNA extracted. Cells were then differentiated for a further 14 days, 11 β -HSD1 activity measured, total RNA extracted and H6PDH and 11 β -HSD1 expression measured by real-time PCR as described below.

Transient transfection of chinese hamster ovary (CHO) cells

Full length cDNA encoding rat 11 β -HSD1 was subcloned into pDR2 (Clontech). The pDR2 derivative encoding human 11 β -HSD2 cDNA has been previously described (Brown *et al.* 1996). The coding region of human H6PDH cDNA (Mason *et al.* 1999) was subcloned into pcDNA3.1 (Invitrogen). PBADHuG6PD, encoding human G6PD (Persico *et al.* 1986), was kindly provided by Dr P Mason (Imperial College, London, UK). Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin. Twenty-four hours prior to transfection, CHO cells were seeded at 2×10^5 cells/60 mm dish and transfected with a total of 21 μ g DNA using the calcium phosphate procedure (Voice *et al.* 1996). DNA consisted of 10 μ g pDR2 vector or 11 β -HSD expression plasmid, 10 μ g pcDNA3.1, H6PDH or pBADHuG6PD plasmid and 1 μ g pCH110 (Amersham) encoding β -galactosidase; the last plasmid was an internal control for transfection efficiency. In each experiment, transfections were independently carried out in sextuplet; three for each assay of 11 β -HSD activity in oxo-reductase or dehydrogenase direction after adding ³H-steroid to the medium 2 h after transfection. Forty two hours after transfection, cells were lysed and β -galactosidase activity assayed, as previously described (Williams *et al.* 2000), to verify that transfection efficiency was similar between transfections.

siRNA experiments

HEK 293T1 cells were used for siRNA transfection. They were derived from human embryonic kidney cells (HEK 293) by stable transfection with human 11 β -HSD1 cDNA in the vector pCR3 (Invitrogen, UK) and display both dehydrogenase and oxo-reductase activities (Bujalska *et al.* 1997a). Cells were plated at 3.0×10^4 cells per well in a 24-well tissue culture plate. Following 24 h in culture, cells were transfected with 100 nM H6PDH siRNA (Ambion siRNA ID 14371), 11 β -HSD1 siRNA (Ambion siRNA ID 16305), or Silencer™ Negative Control #1 siRNA (Ambion, Huntingdon, Cambs, UK) using 3 μ l per well siPORT Amine Transfection Agent (Ambion) according to the manufacturer's protocol. Forty eight hours after transfection, 11 β -HSD1 assays were performed as described below and the cells were subsequently harvested for protein and total RNA. H6PDH and 11 β -HSD1 mRNA levels were measured by real-time RT-PCR with 18S rRNA as the internal standard. Target gene expression was calculated as fold change compared with the negative control siRNA gene expression. All experiments were carried out in triplicate.

RNA extraction and reverse transcription

Total RNA was extracted using a single step extraction method (Tri Reagent, Sigma). One microgram of total RNA was reverse transcribed using random hexamers in 20 μ l volume according to manufacturer's protocol (Promega).

Real-time PCR

11 β -HSD1 and H6PDH mRNA levels were measured by real-time PCR using an ABI 7700 system (Perkin-Elmer, Biosystems, Warrington, UK). PCR was performed in 25 μ l reactions on 96-well plates. Reactions contained TaqMan universal PCR master mix (Applied Biosystems, Warrington, Cheshire, UK), 900 nmol primers, 100–200 nmol TaqMan probe and 25–50 ng cDNA. All reactions were multiplexed with primers specific for 18S rRNA (provided as a pre-optimized mix; Perkin-Elmer, Beaconsfield, Bucks, UK) as an internal reference. All target gene probes were labeled with the fluorescent label FAM, and the 18S probe with the fluorescent label VIC. Reactions were as follows: 50 °C for 2 min, 95 °C for 10 min, and then 44 cycles of 95 °C for 15 sec and 60 °C for 1 min. Data were analyzed according to the manufacturer's guidelines and were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine dCt values ($dCt = Ct$ of the target gene minus Ct of the internal reference, 18S).

Sequences of oligonucleotide primers and probes for 11 β -HSD1 and H6PDH were as follows; 11 β -HSD1: forward 5'-AGGAAAGCTCATGGGAGGACTAG-3', reverse 5'-ATGGTGAATATCATCATGAAAAAGATTTC-3', and probe 5'-CATGCTCATTCTCAACCACATCACCAACA-3'. H6PDH: forward 5'-AGAACTCGGGACCTTTTTC-3', reverse 5'-GCGCCACAGCCTGCTT-3', and probe 5'-TAAGTAATGGTCCACCCGGTACATCTCCTCC-3'.

Western blot analysis

Twenty micrograms of protein from cell lysates (siRNA experiments) was analysed by SDS-PAGE using 12.5% gels on a Bio-Rad Mini-Protein II apparatus (Bio-Rad, Richmond, CA, USA). Ten micrograms of human liver homogenate, produced as previously reported (Ricketts *et al.* 1998), was loaded as a control. Western blotting was performed as reported previously (Ricketts *et al.* 1998). Membranes were incubated with validated polyclonal antibody to human 11 β -HSD1 (The Binding Site, Birmingham, UK) at a dilution of 1:1000 for 16 h at 4 °C. Following 3×10 min washes in PBS-containing 0.1% Tween 20, membranes were incubated with secondary antibody (goat anti-sheep IgG peroxidase conjugate; The Binding Site, Birmingham, UK) at a

dilution of 1:75 000 for 1.5 h at room temperature. Bound peroxidase-conjugated Ig was visualized using ECL detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) by exposing membranes to X-ray film (Kodak).

11 β -hydroxysteroid dehydrogenase assay

Assays of 11 β -HSD1 activity were performed by incubating intact cells with 1 ml of serum-free culture media with 100 nM cortisone (oxo-reductase) or cortisol (dehydrogenase) and tritiated tracer for 3–16 h, or (for transfected CHO cells) with 25 nM corticosterone or 11-dehydrocorticosterone for 42 h. After incubation, media was transferred to a glass tube and steroids were extracted with 5 ml of dichloromethane. Next the aqueous phase was removed and dichloromethane phase concentrated to 100 μ l which was then spotted on a TLC silica plate. Steroids were separated by thin layer chromatography using a mobile phase of ethanol and chloroform (8:92) and quantitated using a Bioscan 2000 image analyzer (Lablogic, Sheffield, UK) or a phosphorimager (Fuji FLA-2000, Raytek Scientific Ltd, Sheffield, UK). Protein levels were assayed using a 96-well plate assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). All experiments were carried out in triplicate.

Data analysis

Values are given as means $\pm$ S.E. of (n) separate experiments. Statistical analysis of comparisons among groups was undertaken using the one-way analysis of variance (for normal distribution) or Mann–Whitney rank sum test (for non-normal distribution). Non-parametric Spearman correlation has been used to analyze preadipocyte data. P values of less than 0.05 were accepted as statistically significant.

Results

Overexpression of H6PDH, G6PD and 11 β -HSD in CHO cells

In transfected cell lines, 11 β -HSD1 is predominantly an oxo-reductase (Low *et al.* 1994). The important exception to this is CHO cells, in which transiently transfected 11 β -HSD1 encodes both oxo-reductase and dehydrogenase activities at approximately equal levels (Agarwal *et al.* 1989). Co-transfection of CHO cells with an expression plasmid encoding H6PDH together with an 11 β -HSD1 plasmid increased oxo-reductase activity compared with controls transfected with 11 β -HSD1 plasmid and empty pcDNA3.1 vector; 68.7 ± 3.0 vs $42.4 \pm 2.4\%$ respectively, $P < 0.05$ (Fig. 1A). Simultaneously, dehydrogenase activity was virtually eliminated; 4.0 ± 0.7 vs $27.2 \pm 1.9\%$ in controls,

Figure 1 Oxo-reductase (A) and dehydrogenase (B) activity of two isozymes of 11 β -HSD. CHO cells were co-transfected with 11 β -HSD1 or 11 β -HSD2 and H6PDH or G6PD. * $P < 0.05$.

$P < 0.05$ (Fig. 1B). In contrast, H6PDH had no effect on reaction direction of 11 β -HSD2; $0.8 \pm 0.3\%$ vs $1.2 \pm 0.5\%$ in controls (oxo-reductase) and $97.5 \pm 1.6\%$ vs $91.0 \pm 5.2\%$ in controls (dehydrogenase) (Figs 1A and B). Furthermore co-transfection of CHO cells with an expression plasmid encoding the cytosolic enzyme G6PD failed to produce any differences in oxo-reductase ($32.7 \pm 2.4\%$ vs controls $42.4 \pm 2.4\%$) or dehydrogenase ($19.0 \pm 1.2\%$ vs controls $27.2 \pm 1.9\%$) activities (Figs 1A and B).

Suppression of 11 β -HSD1 and H6PDH in HEK 293T1 cells by siRNA

Introduction of either 11 β -HSD1 or H6PDH siRNA in HEK 293T1 cells caused a significant decrease in the target mRNA levels compared with cells transfected with a control/scrambled siRNA (Silencer Negative Control #1); 11 β -HSD1 mRNA fold change: 0.48 ± 0.07 , $n=6$, $P < 0.01$, H6PDH mRNA fold change: 0.46 ± 0.12 , $n=6$, $P < 0.01$, (Fig. 2A). Furthermore, 11 β -HSD1 protein levels were significantly decreased in cells transfected with 11 β -HSD1 siRNA compared with cells transfected with H6PDH siRNA or a scrambled siRNA control (Fig. 2B). Both 11 β -HSD1 dehydrogenase and oxo-reductase activities were similarly decreased in intact cells from 11 β -HSD1 siRNA-transfected cells compared with scrambled siRNAs: dehydrogenase; 0.64 ± 0.11 , $n=6$, $P < 0.001$, and oxo-reductase; 0.85 ± 0.14 , $n=6$, $P < 0.05$ (Fig. 2C).

In contrast, transfection with H6PDH siRNA had no effect on 11 β -HSD1 protein levels (Fig. 2B), but did cause a significant decrease in oxo-reductase activity in intact cells compared with cells transfected with scrambled siRNA; fold change 0.81 ± 0.13 , $n=6$, $P < 0.05$, (Fig. 2C). Of note, 11 β -HSD1 dehydrogenase activity was concomitantly increased by transfection with H6PDH siRNA, fold change 1.11 ± 0.09 , $n=6$, $P < 0.05$ (Fig. 2C).

Correlation between 11 β -HSD1 activity and H6PDH expression in human omental preadipocytes

To investigate whether H6PDH similarly regulates 11 β -HSD1 reaction direction in intact primary cultures endogenously expressing 11 β -HSD1, oxo-reductase activity and H6PDH mRNA levels were assayed in human omental preadipocytes. Significant oxo-reductase activity was seen in all cultured preadipocyte preparations.

A significant ($P < 0.05$) negative correlation between dCt for H6PDH [Median 11.04; 10.64 (25%), 11.6 (75%) CI] and 11 β -HSD1 oxo-reductase activity [Median 37.0; 21.02 (25%), 59.60 (75%) CI] in omental human preadipocytes was observed. This illustrates a positive correlation between H6PDH mRNA and 11 β -HSD1 oxo-reductase activity. To present the positive directionality of the correlation, H6PDH mRNA levels have been expressed as $-dCt$, and its relation with 11 β -HSD1 oxo-reductase has been shown

Figure 2 Suppression of 11 β -HSD1 and H6PDH in HEK 293T1 cells by siRNA. (A) HEK 293T1 were transfected with 11 β -HSD1, H6PDH or scrambled-siRNAs (Negative) for 48 h. Compared to scrambled siRNA control, mRNA levels for 11 β -HSD1 and H6PDH were suppressed in cells transfected with the respective siRNAs. (B) Immunoblot showing suppression of 11 β -HSD1 protein levels by 11 β -HSD1-siRNA but not by H6PDH-siRNA or scrambled-siRNAs (Negative); human liver was used as positive control. (C) Resulting 11 β -HSD1 oxo-reductase and dehydrogenase activities were both reduced by 11 β -HSD1-siRNA whereas oxo-reductase activity was decreased and dehydrogenase activity simultaneously increased upon transfection with H6PDH-siRNA. ** P < 0.01; *** P < 0.001 (one-way ANOVA).

in Fig. 3A. The change in 11 β -HSD1 oxo-reductase levels occurred despite no significant difference in 11 β -HSD1 mRNA levels (dCt: 12.34 $\pm$ 0.25, mean $\pm$ S.E.).

As previously reported in a larger cohort (Tomlinson *et al.* 2002), we observed a significant (P < 0.05) inverse correlation between BMI [Median 29.3; 26.2 (25%),

33.1 (75%) CI] and 11 β -HSD1 oxo-reductase activity [Median 37.0; 21.02 (25%), 59.60 (75%) CI] in cultured preadipocytes (Fig. 3B), but importantly no correlation between BMI and 11 β -HSD1 mRNA levels (P = 0.18) (data not shown).

Moreover, H6PDH mRNA levels inversely correlated with BMI as reflected by a positive correlation (P = 0.012) between dCt for H6PDH expression [Median 11.04; 10.64 (25%), 11.6 (75%) CI] and BMI [Median 29.3; 26.2 (25%), 33.1 (75%) CI]. Again, H6PDH mRNA levels have been expressed as -dCt and the correlation between -dCt and BMI has been shown in Fig. 3C.

Across adipocyte differentiation, 11 β -HSD1 oxo-reductase activity increased (11.8 $\pm$ 3.6 vs 66.1 $\pm$ 21.9 pmol/mg/h, day 1 vs day 14, P < 0.05) whilst dehydrogenase activity decreased (104.0 $\pm$ 25.1 vs 17.3 $\pm$ 5.0 pmol/mg/h, day 1 vs day 14, P < 0.01), (Fig. 4A). Concomitantly, H6PDH mRNA levels increased 5.3-fold as measured by quantitative real-time PCR (dCt 13.5 $\pm$ 0.7 vs 11.5 $\pm$ 0.5, day 1 vs day 14, P < 0.05) (Fig. 4B). There was no significant difference in 11 β -HSD1 mRNA expression during omental preadipocytes differentiation (Fig. 4B).

Discussion

The oxo-reductase activity of 11 β -HSD1 has emerged as an important mechanism by which glucocorticoid concentrations can be regulated in a tissue-specific fashion. By generating cortisol in hepatocytes (Jamieson *et al.* 1995, Ricketts *et al.* 1998), adipocytes (Bujalska *et al.* 1997b), osteoblasts (Bland *et al.* 1999, Cooper *et al.* 2000) and ocular tissues (Rauz *et al.* 2001), this enzyme may be involved in the pathogenesis of obesity, metabolic syndrome/diabetes mellitus, glucocorticoid-induced osteoporosis and glaucoma. In liver for example, 11 β -HSD1-mediated oxo-reductase activity stimulates hepatic gluconeogenesis in fasted mice (Kotelevtsev *et al.* 1997). Mice lacking 11 β -HSD1 resist hyperglycemia upon stress or feeding (Kotelevtsev *et al.* 1997). Conversely, transgenic over-expression of 11 β -HSD1 in liver leads to insulin resistance and hypertension, but without obesity (Paterson *et al.* 2004). The role of 11 β -HSD1 in the regulation of adipose tissue mass is an area of much scientific interest and speculation. The profound effects of cortisol upon adipose tissue biology are exemplified in patients with Cushing's syndrome who develop marked and reversible central obesity (Rebuffe-Scrive *et al.* 1988). We have demonstrated enhanced 11 β -HSD1 expression in omental compared with subcutaneous depots, and have suggested that central obesity might arise from the autocrine generation of cortisol within omental adipose tissue – 'Cushing's disease of the omentum' through the expression of

Figure 3 (A) Significant positive correlation between H6PDH mRNA levels and 11 β -HSD1 oxo-reductase activity in human omental preadipocytes. (B) 11 β -HSD1 oxo-reductase activity in omental preadipocytes in relation to BMI from 15 female patients, showing a significant negative association. (C) H6PDH mRNA expression versus BMI from 15 female patients, showing a significant negative correlation.

Figure 4 (A) Change from 11 β -HSD1 dehydrogenase activity to oxo-reductase in human omental preadipocytes differentiated for 14 days, $n=12$. (B) Increase in H6PDH mRNA expression (but not in 11 β -HSD1) concomitant with an increase in oxo-reductase activity in differentiated preadipocyte cultures, $n=12$. * $P<0.05$; ** $P<0.01$.

11 β -HSD1 (Bujalska *et al.* 1997b). However, subsequent studies have shown, if anything, a 10–20% global reduction in 11 β -HSD1 expression in obese subjects, although expression within adipose tissue in obesity is still debated (Stewart *et al.* 1999, Tomlinson *et al.* 2002). Nevertheless, recent animal data have highlighted the beneficial effects of administration of selective 11 β -HSD1 inhibitors upon insulin sensitivity and body weight (Alberts *et al.* 2003). The effects of 11 β -HSD1 upon adipose tissue biology depend upon site of expression and enzyme directionality. Cortisol inhibits proliferation of preadipocytes yet stimulates differentiation to adipocytes (Tomlinson *et al.* 2002). Immediately following isolation of preadipocytes, dehydrogenase activity predominates (Bujalska *et al.* 2002) which would enhance proliferation. However, once established in cell culture, activity is predominantly oxo-reductase (Bujalska *et al.* 1997b) and serves to promote adipogen-

esis (Bujalska *et al.* 1999). This is most elegantly demonstrated in transgenic mice over-expressing 11 β -HSD1 under the adipocyte specific aP2 promoter who develop central obesity as a consequence of increased adipocyte size (Masuzaki *et al.* 2001). Determining the factors regulating directionality of 11 β -HSD1 activity in human adipose tissue is therefore of crucial importance. In other tissues, 11 β -dehydrogenase activity has been reported in intact cell preparations with the direction of 11 β -HSD1 catalysis appearing to vary according to physiological or developmental status of a particular cell type. In Leydig and neuronal cells, as well as in human bone, both 11 β -dehydrogenase and oxo-reductase activities have been observed (Ge *et al.* 1997, Leckie *et al.* 1998, Jellinck *et al.* 1999, Cooper *et al.* 2000). Only oxo-reductase activity has been detected in kidney cells of monkey origin (Cos-7) transiently transfected with rat 11 β -HSD1 cDNA (Low *et al.* 1994). However, in most

cell models, stably transfected with human 11 β -HSD1 cDNA into HEK 293 cells (Bujalska *et al.* 1997a) or transiently transfected with rat 11 β -HSD1 into CHO cells (Agarwal *et al.* 1989), both oxo-reductase and dehydrogenase activity are reported. These studies have used different cell models and cDNA/expression constructs that might explain data inconsistency. Yet in every case where oxo-reductase activity is identified, it is reduced or lost when cells are disrupted or the enzyme purified (Monder & Lakshmi 1989, Monder *et al.* 1991). This striking change in directionality between intact cells and homogenates is apparently a consequence of the specific intracellular localization of 11 β -HSD1 within the lumen of the ER (Ozols 1995), where H6PDH generates the reduced co-substrate, NADPH (Ozols 1993). Although NADPH generation within the ER lumen is potentially of critical importance to cellular function, at present there is little known about the cellular localization of the catalytic domains of other ER reductases, with the exception of 11 β -HSD1. H6PDH is present at high levels within tissues known to express 11 β -HSD1 (Beutler & Morrison 1967), and blocking the transport of glucose-6-phosphate into isolated rat liver microsomes inhibits both H6PDH and 11 β -HSD1 activities (Gerin & Van Schaftingen 2002, Banhegyi *et al.* 2004). These findings complement the observation that patients with CRD have functional mutations in *H6PD* in association with intronic mutations in *HSD11B1* that attenuate 11 β -HSD1 expression (Draper *et al.* 2003).

Here we show unequivocally that H6PDH is able to regulate 11 β -HSD1 enzyme direction. Co-expression of 11 β -HSD1 with H6PDH in CHO cells increased oxo-reductase activity whilst reducing dehydrogenase activity. A parallel study (Atanasov *et al.* 2004) also showed a 6-fold increase in oxo-reductase with simultaneous 6-fold decrease in dehydrogenase in transiently transfected HEK 293 cells with 11 β -HSD1 and H6PDH, confirming our earlier concept of a close inter-relationship between these two enzymes within the ER. In this study, we have further shown the specificity of 11 β -HSD1 for H6PDH, since co-transfection with a cytosolic NADPH-provider, G6PD, failed to modulate 11 β -HSD1 reaction direction. In contrast, the overwhelmingly oxidative reaction direction of 11 β -HSD2 was unaffected by co-transfection with H6PDH, as predicted given that the active site of this enzyme faces toward the cytosol (Naray-Fejes-Toth & Fejes-Toth 1996). This finding is indicative of a pool of co-factors localized within the ER lumen, separated from the cytosolic compartment.

Additionally, we have further endorsed these findings through siRNA 'knock-down' experiments in HEK 293T1 cells, where a 50% reduction in 11 β -HSD1 or H6PDH mRNA expression was achieved. Reduction in 11 β -HSD1 mRNA altered dehydrogenase and oxo-reductase activities to a similar degree, with the

concurrent decrease in 11 β -HSD1 protein expression. Silencing of H6PDH, however, preferably reduced oxo-reductase activity whilst increasing dehydrogenase activity.

Here, we have shown an increase in oxo-reductase 11 β -HSD1 activity and H6PDH expression in differentiating human omental preadipocytes. In a parallel study, using the 3T3-L1 mouse cell line, a modest increase in H6PDH mRNA levels has been shown to occur across adipogenesis, associated with a highly significant increase in 11 β -HSD1 oxo-reductase activity (Atanasov *et al.* 2004). However, in this cell system, levels of 11 β -HSD1 mRNA also increased more than 100-fold across adipogenesis. This alone could have accounted for the observed significant increase in 11 β -HSD1 oxo-reductase activity which is not the case in our human cell model where the increase in 11 β -HSD1 oxo-reductase occurred despite unchanged levels of 11 β -HSD1 expression.

In addition, in this study, we demonstrate that expression H6PDH mRNA levels decreased with increasing BMI, perhaps explaining the observed decrease in 11 β -HSD1 oxo-reductase activity in omental preadipocytes in obese patients (Tomlinson *et al.* 2002). Our earlier studies have shown that glucocorticoids inhibit preadipocyte proliferation (Tomlinson *et al.* 2002); thus the impaired oxo-reductase activity may increase the number of proliferating preadipocytes in visceral depots. Because 11 β -HSD1 mRNA levels in human omental adipose tissue are poorly correlated with BMI (Tomlinson *et al.* 2002), we hypothesize that the 'set-point' of 11 β -HSD1 reaction direction may be contributing more to the pathogenesis of central obesity than its absolute expression levels. On this note, it is interesting to speculate that earlier studies that inferred increased oxo-reductase activity in obese subjects, based upon an increase in measured dehydrogenase activity, might not represent the *in vivo* situation as this reflects the amount of enzyme present but not its directionality (Rask *et al.* 2001, Wake *et al.* 2003). To summarize, in this study we now demonstrate a direct relationship between 11 β -HSD1 oxo-reductase activity and H6PDH expression in human omental preadipocytes and cell line models.

In terms of human disease, the targeted inhibition of 11 β -HSD1 as a therapeutic strategy in patients with the metabolic syndrome is an exciting advance (Alberts *et al.* 2002, Andrews *et al.* 2003, Sandeep *et al.* 2004, Rauz *et al.* 2003). In turn, the identification of H6PDH as a determinant of oxo-reductase activity in adipose tissue raises the possibility that H6PDH itself could be a promising therapeutic target in treating central obesity. Indeed it is tempting to speculate that some of the 'selective 11 β -HSD1' inhibitors obtained through cell-based high throughput screening assays, might actually be inhibitors of H6PDH rather than 11 β -HSD1.

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