Hexose-6-phosphate dehydrogenase determines the reaction direction of 11β-hydroxysteroid dehydrogenase type 1 as an oxoreductase

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Abstract The impact of hexose-6-phosphate dehydrogenase (H6PDH) on 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 activity was investigated upon coexpression in HEK-293 cells. Confocal microscopy analysis indicated colocalisation of both enzymes at the lumenal side of the endoplasmic reticulum (ER) membrane. Functional analysis in intact cells revealed fivefold stimulation of 11β-HSD1 oxoreductase activity and sixfold decrease of dehydrogenase activity upon coexpression with H6PDH, without changing kinetic parameters in cell lysates. Thus, H6PDH directly determines the reaction direction of 11β-HSD1 in intact cells as an oxoreductase without changing intrinsic catalytic properties of 11β-HSD1 by regenerating NADPH in the ER-lumen.

Keywords: 11β-Hydroxysteroid dehydrogenase; Hexose-6-phosphate dehydrogenase; Glucocorticoid; Cortisol

1. Introduction

Cortisone, an inactive prohormone, requires the oxoreduction of its 11-keto group into an 11-hydroxyl group to form biologically active cortisol. The ratio of cortisone to cortisol is controlled by 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes (reviewed in [1]). 11β-HSD2 exclusively catalyses the oxidation of cortisol to cortisone, thereby protecting the mineralocorticoid receptor from cortisol and rendering specificity for aldosterone in tissues such as kidney and colon. 11β-HSD1 is a bidirectional enzyme, catalysing both oxidation and oxoreduction with comparable efficiencies in cell lysates, whereby oxoreduction is less stable and decreases upon preparation of microsomes and purification [2]. Experiments in animals and humans indicated that 11β-HSD1, which is highly expressed in liver and adipose tissue [1] and has been associated with obesity and type 2 diabetes mellitus [3], is predominantly an oxoreductase in vivo [4–7].

Oxidation of glucocorticoids was also reported in cells devoid of 11β-HSD2 including skin fibroblasts [8], rat pituitary GH3 cells [9], Leydig cells [10,11] and adipose stromal cells [12]. Recent reports suggested that hexose-6-phosphate dehydrogenase (H6PDH), catalysing the first two steps of pentose phosphate pathway, may stimulate 11β-HSD1 oxoreduction by generating NADPH in the ER-lumen. Patients with cortisone oxoreductase deficiency have mutations in both *HSD11B1*, causing decreased expression of 11β-HSD1, and *H6PD*, causing reduced H6PDH activity [13]. Furthermore, a switch from 11β-HSD1 dehydrogenase to oxoreductase activity and an increase in H6PDH mRNA was observed during differentiation of adipose stromal cells to mature adipocytes [12]. However, the differentiation of adipose stromal cells into adipocytes is a highly complex process with dramatic changes in gene expression, making it difficult to assess the relative contribution of H6PDH on 11β-HSD1 function.

In line with a stimulatory effect of H6PDH on 11β-HSD1, Banhegyi et al. [14] demonstrated cooperativity between the two enzymes in liver microsome experiments. However, dehydrogenase activity was not measured and cortisone-dependent stimulation of H6PDH activity was observed at 10–500 μM cortisone, concentrations far from being physiological. Thus, the use of liver microsomes to assess the physiological role of H6PDH on 11β-HSD1 function is limited.

The present study aimed at the reconstitution of the effects of H6PDH on 11β-HSD1 activities in intact cells with no or very low endogenous expression of these two enzymes.

2. Materials and methods

2.1. Materials

Cell culture media and reagents for reverse transcription of RNA were from Invitrogen (Carlsbad, CA), [1,2,6,7-^{3}H]-cortisol from Amersham Bioscience (Piscataway, NJ), [1,2,6,7-^{3}H]-cortisone from American Radiolabelled Chemicals (St. Louis, MO), the SV total RNA isolation system from Promega (Madison, WI) and reagents for TaqMan real-time PCR from Applied Biosystems (Foster City, CA). Monoclonal antibodies were from Roche Diagnostics (Rotkreuz, Switzerland) and fluorescent antibodies from Molecular Probes (Eugene, OR).

2.2. Construction of plasmids

The expression plasmid for C-terminally FLAG-tagged human 11β-HSD1 was described previously [15]. Full length human hexose-6-phosphate dehydrogenase cDNA was kindly provided by Dr. P.J. Mason (Accession No. AJ012590 [16]). The H6PDH cDNA was modified by introducing an *Eco*RI site and by changing the context 5′ to the initiator ATG codon to a ''Kozak'' consensus sequence [17] after PCR amplification with the oligonucleotide 5′-TGTGAATTCC-GCCATGTGGAATATGCTCATAGTGGC-3′. An *Xba*I site was introduced immediately 3′ of the stop codon with oligonucleotide

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Abbreviations: 11β-HSD, 11β-hydroxysteroid dehydrogenase; ER, endoplasmic reticulum; H6PDH, hexose-6-phosphate dehydrogenase

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5′-ATATCTAGATCATCCCAGGAAGGCGTCGTAG-3′ or a C-terminal c-myc-epitope (-EQKLISEEDL-COOH), separated from the H6PDH sequence by a SGGS-linker, with oligonucleotide 5′-ATATCTAGATCAGAGGTCTTCTTCTGAGATGAGTTTTTGT-TCTGATCCTCCTGATCCCAGGAAGGCGTCGTAG-3′. The PCR product was sequenced and cloned into pcDNA3 expression vector (Invitrogen) with *Eco*RI and *Xba*I.

2.3. Cell culture and transient transfection

HEK-293 cells were cultured as described [18] and transfected at a 1:1 ratio with 11β-HSD1 and either H6PDH or control DNA (pcDNA3 vector) using the Ca^{2+}-phosphate precipitation method. Chinese hamster ovary (CHO) cells were grown in Ham's-F12 medium and transfected with FuGENE6 according to the manufacturer's manual (Roche). After 24 h, cells were incubated in steroid-free medium for another 24 h, lysates prepared and activities determined. For determination of enzyme activities in intact cells, 80 000 cells were seeded per well on a 96-well plate at the time of the change to steroid-free medium, followed by incubation for 24 h. 3T3-L1 mouse preadipocytes were cultured and differentiated into adipocytes as described [18].

2.4. RNA extraction, reverse transcription and quantitative PCR

Total RNA was extracted from HEK-293 cells using the SV total RNA isolation system. Reverse transcription was performed in 20 μl containing 2 μg total RNA, 100 U Superscript II reverse transcriptase, 1 μg Oligo$(dT)_{12-18}$, 0.5 mM of each deoxynucleotide, 10 mM dithiothreitol, 40 U RnaseOUT recombinant ribonuclease inhibitor and the manufacturer's buffer (Invitrogen). Real-time quantification of H6PDH mRNA was analysed using an ABI7000 sequence detection system (Applied Biosystems) and TaqMan technology for highly accurate H6PDH mRNA quantitation. Reactions were performed in 96-well plates in 25 μl of reaction buffer containing TaqMan Universal PCR Master Mix and 100 ng cDNA. Quantitative primer and probe sequences are available on request (Assay-on-Demand). All reactions were multiplexed with 18S rRNA control probe. Data were obtained as Ct values according to the manufacturer's guidelines.

2.5. Immunofluorescence analysis

Immunostaining was performed as described [15]. Briefly, cells were fixed with 4% paraformaldehyde, washed, blocked in buffer containing 1% milk and incubated with 0.5% Triton X-100 for complete permeabilisation of membranes or 25 μM digitonin for selective permeabilisation of the plasma membrane. FLAG-tagged 11β-HSD1 was detected using mouse anti-FLAG antibody M2 and ALEXA-488 goat anti-mouse antibody. Myc-tagged H6PDH was detected with rabbit anti-myc antibody and ALEXA-594 goat anti-rabbit antibody. Samples were analysed on an LSM410 confocal microscope (Carl Zeiss, Goettingen, Germany).

2.6. 11β-HSD1 activity assay

For measurements in lysates, cells were washed, resuspended and centrifuged for 3 min at $150\times g$. The supernatant was removed, pellets were quick-frozen in a dry-ice ethanol bath and aliquots stored at −70 °C. 11β-HSD1 oxoreductase and dehydrogenase activities in cell lysates were measured as described [18] in the presence of 400 μM NADPH or NADP, respectively, with substrate concentrations ranging from 20 nM to 2.5 μM. Assays with intact cells were carried out in 96-well plates. Cells were incubated in steroid-free medium in the presence of various substrate concentrations in the absence of exogenous cofactor for 1–3 h at 37 °C. In all reactions, the conversion of substrate to product was kept below 30%. Steroids were separated by TLC and conversion analysed by scintillation counting.

To semi-quantitatively assess V_{max}, 30 μg of total proteins from transfected cells was subjected to SDS–PAGE and Western blot analysis using mouse anti-FLAG antibody M2 to detect FLAG-tagged 11β-HSD1 and mouse anti-myc antibody to detect myc-tagged H6PDH, followed by horseradish peroxidase-conjugated anti-mouse IgG. Antibody binding was visualised using the enhanced chemiluminescence (ECL) Western detection system (Pierce, Rockford, IL).

3. Results and discussion

3.1. Expression analysis of 11β-HSD1 and H6PDH in 3T3-L1 adipocytes

In line with previous observations [19,20], we reported recently that 11β-HSD1 catalyses exclusively the oxoreduction reaction in intact rat hepatocytes and fully differentiated mouse 3T3-L1 adipocytes [18,21]. In contrast, transfected HEK-293 cells equally well catalysed oxoreduction and oxidation of glucocorticoids [15]. Here, we investigated the direct effect of H6PDH on 11β-HSD1 activities in intact cells.

First, we measured 11β-HSD1 activities and determined mRNA levels of 11β-HSD1 and H6PDH in mouse 3T3-L1 preadipocytes and in fully differentiated adipocytes (Fig. 1). While only background 11β-HSD1 activity was detectable in 3T3-L1 preadipocytes after incubation for 3 h (or even after 12 h, not shown), fully differentiated 3T3-L1 adipocytes efficiently catalysed the oxoreduction of cortisone (Fig. 1A), resembling the activities in mature adipocytes. The increase in 11β-HSD1 activity upon differentiation to 3T3-L1 adipocytes was reflected by a more than 100-fold increase in 11β-HSD1 mRNA in fully differentiated adipocytes (Fig. 1B). In contrast, H6PDH mRNA only slightly increased during 3T3-L1 adipocyte differentiation, reaching 2.5-fold higher levels seven days after initiation of differentiation. This stands in contrast to the differentiation of human adipose stromal cells to adipocytes, where 11β-HSD1 mRNA did not change but H6PDH mRNA increased twofold [13]. Thus, due to the lack of substantial 11β-HSD1 expression, 3T3-L1 preadipocytes are

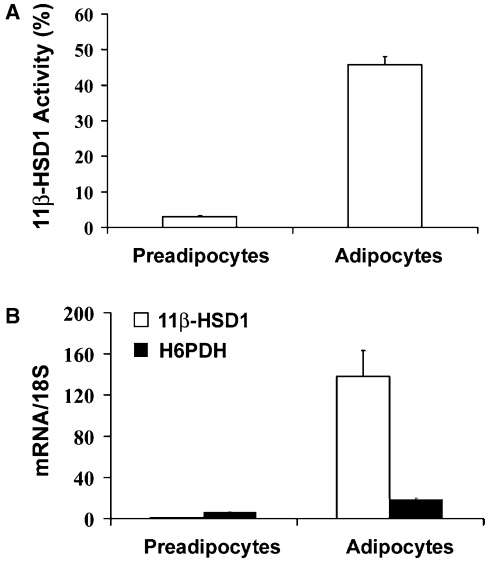

Fig. 1. Activity of 11β-HSD1 and expression of 11β-HSD1 and H6PDH mRNA in 3T3-L1 preadipocytes and in fully differentiated adipocytes. Cells were grown to confluence as preadipocytes or were differentiated into adipocytes by incubation with isobutylmethylxanthine, dexamethasone, and insulin for two days followed by incubation in medium containing insulin for two more days and in absence of insulin for another three days. (A) 11β-HSD1 activity was determined as the percent conversion of cortisone to cortisol in intact cells incubated for 3 h in medium containing [^{3}H]cortisone. (B) 11β-HSD1 (white bars) and H6PDH mRNA levels (black bars) relative to 18S control RNA were determined by quantitative real time TaqMan PCR. Data were normalized to the ratio of 11β-HSD1 mRNA to 18S RNA in preadipocytes. Data represent means ± S.D. from three independent experiments.

not a suitable system to study the effects observed in adipose stromal cells. The complex changes in protein expression during adipocyte differentiation make it difficult to dissect a potential effect of H6PDH on 11β-HSD1 activity from that of other proteins. Therefore, we reconstituted the effects of H6PDH on 11β-HSD1 activities by expressing recombinant enzymes in a cell system with no or very low endogenous levels.

3.2. Expression of recombinant 11β-HSD1 and H6PDH in HEK-293 cells

PCR analysis revealed no endogenous 11β-HSD1 (not shown) and only a very low level of H6PDH mRNA in HEK-293 cells (Fig. 2A). Expression of recombinant H6PDH and 11β-HSD1 resulted in a more than 1000-fold increase in mRNA level (Fig. 2A, not shown). As expected, the expression of C-terminally myc-tagged H6PDH yielded a band at 90 kDa [16]. Unspecific low molecular weight bands in control cells and in H6PDH-transfected cells indicate that equal protein amounts were separated by SDS–PAGE. Coexpression of both proteins resulted in a decreased expression (37%) of 11β-HSD1 protein as determined by densitometry (Fig. 2B).

3.3. 11β-HSD1 and H6PDH colocalise at the lumenal side of the ER-membrane

The intracellular distribution of FLAG-tagged 11β-HSD1 and myc-tagged H6PDH was analysed in transfected HEK-293 cells after selectively permeabilising the plasma membrane using 25 μM digitonin. No signal was detected for both enzymes; in contrast, using the same conditions, control cells expressing FLAG-tagged 11β-HSD2 with cytoplasmic orientation stained positive (not shown, [15]). These results confirm that 11β-HSD1 and H6PDH are ER-lumenal proteins. Dual staining of completely permeabilised cells (0.5% Triton X-100) and analysis by confocal microscopy revealed a nearly identical, typically reticular distribution pattern of 11β-HSD1 and H6PDH (Fig. 3). An overlay image of the expression of H6PDH and 11β-HSD1 indicated colocalisation of both proteins at the ER-membrane. Although H6PDH was originally purified from liver microsomal preparations [22] and was found to be firmly bound to the ER-membrane upon washing and trypsin digestion [23], H6PDH does not contain a membrane spanning region nor was the protein known to which it is associated. Clearly, further studies are required to assess

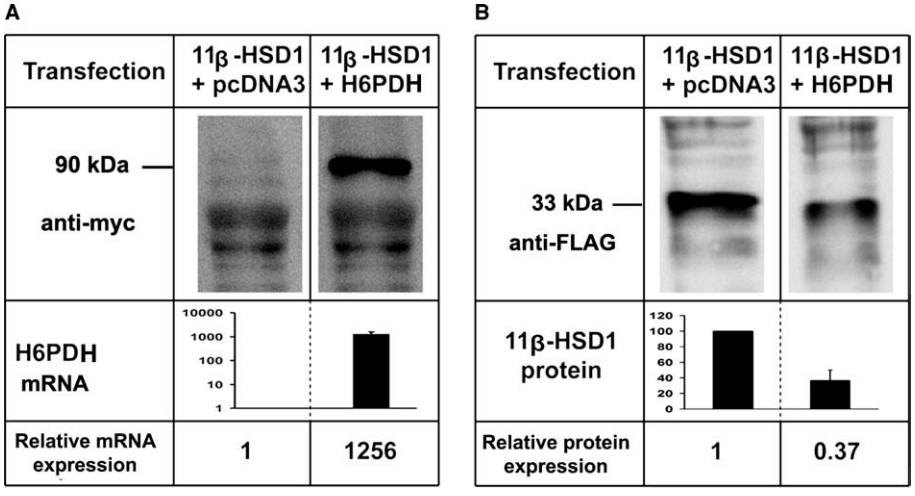

Fig. 2. Expression of 11β-HSD1 and H6PDH in HEK-293 cells. Cells were transfected with the respective constructs, grown for 48 h and 30 μg of total proteins separated by SDS–PAGE. (A) Detection of myc-tagged H6PDH by Western blot and relative quantification of mRNA by TaqMan PCR analysis. (B) Detection of FLAG-tagged 11β-HSD1 by Western blot and relative quantification of Western blot signals (performed by ImageJ software, NIH, USA) (data represent three independent experiments).

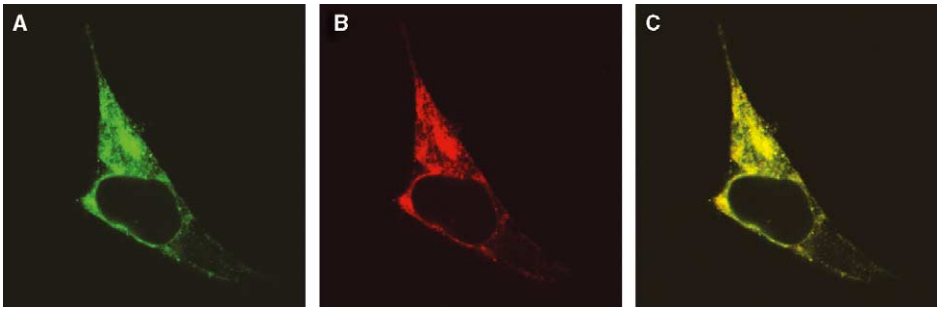

Fig. 3. Colocalisation of 11β-HSD1 and H6PDH to the ER-membrane. HEK-293 cells were cotransfected with FLAG-tagged 11β-HSD1 and myc-tagged H6PDH. After 48 h, cells were completely permeabilised with 0.5% Triton X-100, dual-labelled with mouse anti-FLAG antibody and rabbit anti-myc antibody and visualised using anti-mouse antibody ALEXA-488 and anti-rabbit antibody ALEXA-594. Samples were analysed by confocal microscopy. (A) Green fluorescence representing 11β-HSD1; (B) red fluorescence showing H6PDH; and (C) an overlay of (A) and (B).

Table 1
Effect of H6PDH on 11β-HSD1-dependent interconversion of cortisone and cortisol

Transfection	Oxoreduction of cortisone			Oxidation of cortisol		
	K_m (nM)	V_{max} (nmol h^{-1} mg^{-1})	k_{cat} (V_{max}/K_m) (h^{-1} $mg^{-1} \times 10^{-3}$)	K_m (nM)	V_{max} (nmol h^{-1} mg^{-1})	k_{cat} (V_{max}/K_m) (h^{-1} $mg^{-1} \times 10^{-3}$)
(A) Intact cells						
11β-HSD1 + pcDNA3	665 ± 91	0.76 ± 0.08	1.14	1093 ± 185	1.74 ± 0.3	1.59
11β-HSD1 + H6PDH	862 ± 142	4.57 ± 0.35	5.30	1103 ± 272	0.26 ± 0.07	0.24
11β-HSD1 + H6PDH-myc	807 ± 128	4.21 ± 0.21	5.22	1317 ± 123	0.37 ± 0.08	0.28
(B) Cell lysates						
11β-HSD1 + pcDNA3	295 ± 70	0.58 ± 0.05	1.97	996 ± 206	2.7 ± 0.64	2.71
11β-HSD1 + H6PDH	254 ± 88	0.44 ± 0.06	1.73	1286 ± 213	3.67 ± 0.55	2.85
11β-HSD1 + H6PDH-myc	352 ± 42	0.51 ± 0.05	1.45	1203 ± 243	3.2 ± 0.6	2.66

HEK-293 cells were transfected with 11β-HSD1 and either wild-type H6PDH, C-terminally myc-tagged H6PDH or control DNA. After growth in steroid-free medium for 24 h, activities were determined in intact cells (A) or lysates (B). Activities (means ± S.D.) were obtained from three independent transfections, each measured in duplicate. Kinetic parameters were obtained by non-linear regression using Data Analysis Toolbox (MDL Information System Inc.) assuming first-order rate kinetics. For calculation of apparent V_{max}, the amount of 11β-HSD1 protein was compared by densitometric analysis of Western blots.

whether 11β-HSD1 tethers H6PDH to the ER-membrane by a direct physical interaction.

3.4. H6PDH determines the reaction direction of 11β-HSD1

In the absence of H6PDH, intact HEK-293 cells expressing 11β-HSD1 catalysed the oxoreduction of cortisone and the oxidation of cortisol with a slight preference for the dehydrogenase reaction (Table 1, Fig. 4), making it a suitable system to investigate the mechanisms affecting reaction direction. The ratio between oxoreductase and dehydrogenase activity was comparable between intact cells (0.72) and cell lysates (0.73) in the absence of H6PDH (Table 1). Coexpression with H6PDH led to a fivefold increase in oxoreductase activity and a sixfold decrease in dehydrogenase activity without changing

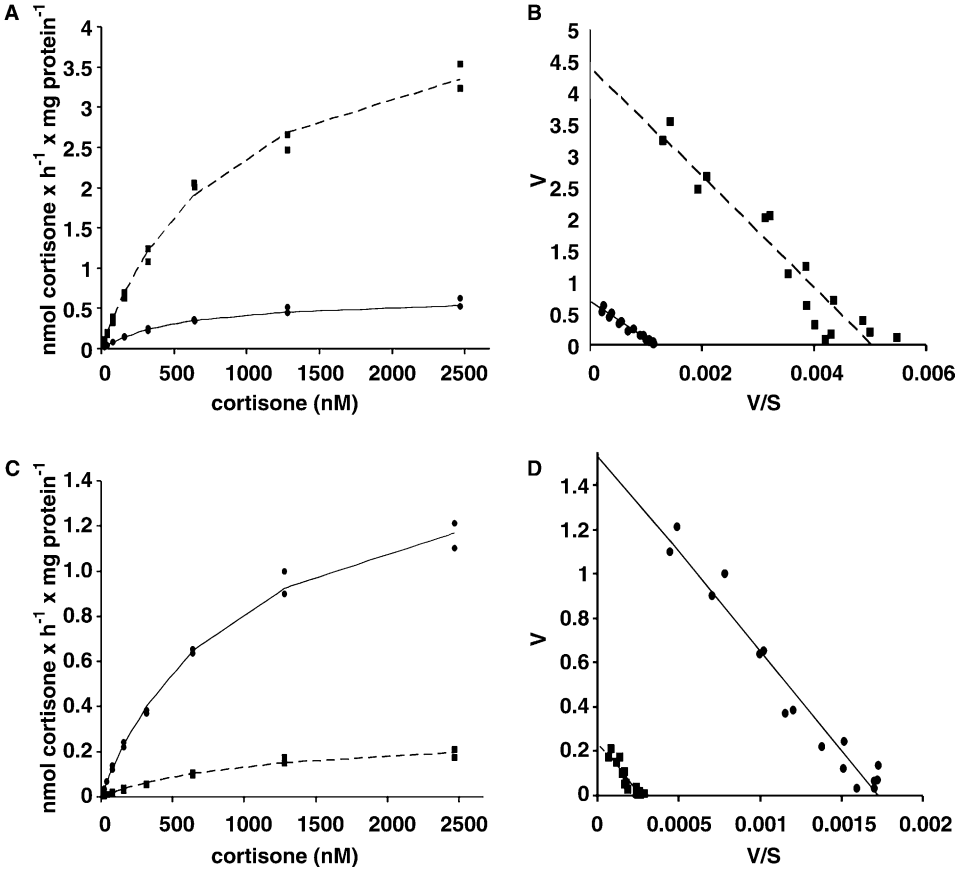

Fig. 4. H6PDH determines the reaction direction of 11β-HSD1. HEK-293 cells were transfected with 11β-HSD1 and control pcDNA3 plasmid (solid line, A–D) or cotransfected with 11β-HSD1 and H6PDH (dashed line, A–D). (A,B) Oxoreduction of cortisone; (C,D) oxidation of cortisol. Representative experiments measured in duplicate are shown. (B,D) Eadie–Hofstee plots demonstrate Michaelis–Menten kinetics both in the absence (solid line) or presence of H6PDH (dashed line). The intercept at the *y*-axis indicates V_{max} (B,D). Data represent means ± S.D. obtained from three independent experiments measured in duplicate.

kinetic parameters in cell lysates (Table 1). H6PDH significantly altered V_{max} without affecting K_m for both oxoreductase and dehydrogenase activity, indicating that the availability of the cofactor does not affect the substrate affinity of 11β-HSD1. The ratio between oxoreductase and dehydrogenase activity increased to 22 in intact cells but was not significantly different in cell lysates (0.62) upon coexpression of H6PDH and 11β-HSD1. The linearity of the data observed in Eadie-Hofstee plots shows first-order rate kinetics for 11β-HSD1 both in the absence and presence of H6PDH (Fig. 4B and D).

Only background activities were detectable in cell lysates when cofactor was omitted. In cell lysates, 11β-HSD1 exists in an accessible form in open perinuclear and in multilamellar membranes, whereas the enzyme seems to be inaccessible for the cofactor and/or the substrate in tightly sealed ER-membrane vesicles. Correspondingly, we observed approximately twofold higher total enzymatic activities upon addition of relatively mild detergents such as 0.5% $C_{12}E_8$, octylglucoside or Nonidet P-40 (not shown). This is in line with Banhegyi et al. [14] reporting cooperativity between 11β-HSD1 and H6PDH in the ER-lumen and demonstrating that the activity of both enzymes was latent in intact rat liver microsomal vesicles.

Similar results were obtained with CHO cells. Coexpression with H6PDH did not affect 11β-HSD1 activities in cell lysates. In intact cells, coexpression with H6PDH stimulated 11β-HSD1-dependent oxoreduction of cortisone from $k_{cat} = 1.8 \pm 0.2\ h^{-1}\,mg^{-1} \times 10^{-3}$ ($K_m = 662 \pm 81$ nM, $V_{max} = 1.19 \pm 0.06$ nmol $h^{-1}\,mg^{-1}$) to $k_{cat} = 5.20 \pm 0.41\ h^{-1}\,mg^{-1} \times 10^{-3}$ ($K_m = 818 \pm 97$ nM, $V_{max} = 4.28 \pm 0.29$ nmol $h^{-1} \times mg^{-1}$) and completely abolished the oxidation reaction (from $k_{cat} = 0.60 \pm 0.05\ h^{-1}\,mg^{-1} \times 10^{-3}$, $K_m = 1172 \pm 159$ nM, $V_{max} = 0.71 \pm 0.05$ nmol $h^{-1}\,mg^{-1}$).

In conclusion, these findings indicate that H6PDH cooperates with 11β-HSD1 by regenerating cofactor NADPH without altering the intrinsic enzymatic properties of 11β-HSD1. The analysis of the intracellular distribution of epitope-tagged 11β-HSD1 and H6PDH constructs provides evidence for colocalisation at the lumenal side of the ER-membrane. The fact that activities in lysates in the absence or presence of H6PDH were not significantly different suggests a rather weak or short-lived interaction between 11β-HSD1 and H6PDH. Importantly, the present findings demonstrate for the first time in intact cells that H6PDH not only stimulates oxoreductase activity of 11β-HSD1 but also determines the reaction direction by abolishing the dehydrogenase activity. These results strongly suggest that the increasing expression of H6PDH during the differentiation of adipocytes controls the observed switch from dehydrogenase activity, favouring cell proliferation, to oxoreductase activity, promoting adipocyte differentiation [12]. Like 11β-HSD1, H6PDH and the glucose-6-phosphate transporter in the ER-membrane, supplying the substrate for H6PDH, represent potential targets for therapeutic treatment of type 2 diabetes. Inhibitors of these enzymes should result in diminished local activation of glucocorticoids and are expected to exert anti-diabetic effects.

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