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Cell-based assay for screening 11β-hydroxysteroid dehydrogenase 1 inhibitors

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ABSTRACT

11β-Hydroxysteroid dehydrogenase 1 (11β-HSD1) is primarily responsible for intracellular biosynthesis of active glucocorticoid, and its tissue-specific dysregulation has been implicated in the development of metabolic syndromes. We have developed a cell-based assay for measuring 11β-HSD1 activities using murine skeletal muscle cell line C2C12. We found that the messenger RNA (mRNA) expression of 11β-HSD1 increased on differentiation with enhanced enzyme activity as determined by homogeneous time-resolved fluorescence (HTRF) assay. Carbenoxolone, a well-known 11β-HSD1 inhibitor, exhibited an IC_{50} value similar to that in in vitro microsomal assay (IC_{50} = 0.3 μM). Unlike in vitro microsomal assay, cosubstrate NADPH was not required in the cell-based assay, indicating that viable cells might provide a sufficient amount of endogenous NADPH to catalyze the enzymatic conversion of inactive cortisone to active cortisol. Treatment of C2C12 myotubes with cortisone concentration dependently transactivated and transrepressed glutamine synthase and interleukin-6, respectively, which were abrogated by carbenoxolone or RU-486 (mifepristone), a glucocorticoid receptor antagonist. Accordingly, a newly designed cell-based assay using differentiated skeletal muscle cells would be useful for high-throughput screening of 11β-HSD1 inhibitors as well as for understanding the molecular mechanisms of glucocorticoid action.

Local production of active glucocorticoid is regulated by 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1)[1] expressed mainly in liver, fat, brain, and skeletal muscle [1,2], and the enzymatic action of 11β-HSD1 is a bidirectional dehydrogenase/reductase pathway depending on the level of NADP/NADPH [3]. Because dysregulation of glucocorticoid biosynthesis in tissues appears to be associated with metabolic syndrome, a cluster of cardiovascular risk factors such as visceral obesity, insulin resistance, dyslipidemia, and hypertension [4], several 11β-HSD1 inhibitors have been developed to treat metabolic diseases by pharmaceutical companies [5,6].

To discover novel inhibitors of 11β-HSD1, assay systems appropriate for the evaluation of enzyme activity are a prerequisite. But currently available assay formats have several shortcomings with regard to the enzyme sources and detection methods of enzymatic activities. For the enzyme sources, either liver microsomes or cell lines transfected with recombinant 11β-HSD1 alone or along with the glucocorticoid response element linked to a β-galactosidase reporter gene have been widely used [7]. However, liver microsomes used in in vitro assay have metabolic capacity for several compounds, and a large amount of NADPH as a cofactor is essential to drive predominantly reductase activity in an assay format. On the other hand, although a supply of NADPH is not required in the existing cell-based assays due to the action of hexose 6 phosphate dehydrogenase (H6PDH) in the endoplasmic reticulum, the application of genetically engineered cell lines for screening of 11β-HSD1 inhibitors would be affected by gene transfection efficiency as well as gene expression milieu within the cells.

To date, catalytic activities from in vitro assays have been determined either by radioactivity of labeled substrate cortisone or by liquid chromatography–tandem mass spectrometry (LC–MS/MS) [8,9]. Alternatively, colorimetric readout of reporter gene under the control of glucocorticoid receptor element through complex formation with glucocorticoid receptor (GR) has been employed [7]. But these methods contain several limitations such as the use of radioactive material, expensive instrument/purification techniques, and interference of chemicals with color development. Therefore, a cell-based assay using an endogenous 11β-HSD1 and convenient detection method was pursued for in vitro high-throughput screening (HTS) of inhibitors.

Peripheral tissues such as muscle, liver, and fat are known to express high levels of 11β-HSD1 [2], but its function remains to be defined. In an attempt to develop cell-based assay suitable for

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[1] *Abbreviations used:* 11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; H6PDH, hexose 6 phosphate dehydrogenase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; GR, glucocorticoid receptor; HTS, high-throughput screening; HTRF, homogeneous time-resolved fluorescence; CBX, carbenoxolone; GS, glutamine synthase; IL-6, interleukin-6; DEX, dexamethasone; TNFα, tumor necrosis factor alpha; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; RT–PCR, reverse transcription polymerase chain reaction; UV, ultraviolet; EtBr, ethidium bromide; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; TLC, thin-layer chromatography; mRNA, messenger RNA; NF-κB, nuclear factor–kappa B; AP-1, activated protein 1.

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HTS, we employed murine skeletal muscle cell line C2C12, and examined the expression and activity of 11β-HSD1 in myoblast and differentiated myotubes using homogeneous time-resolved fluorescence (HTRF) assay. Carbenoxolone (CBX), a well-known inhibitor, exhibited a similar IC_{50} (0.3 μM) when compared with that in in vitro microsomal assay. To evaluate biological functions of cortisol produced by 11β-HSD1 in C2C12 cells, the regulation of glucocorticoid-responsive genes such as glutamine synthase (GS) and interleukin-6 (IL-6) was investigated. Recently, it was shown that treatment of C2C12 myoblast with dexamethasone (DEX) inhibits basal and lipopolysaccharide-stimulated levels of IL-6 and tumor necrosis factor alpha (TNFα), whereas DEX induces transcriptional activation of GS, a marker for muscle wasting [10]. Similar to the previous results using DEX and myoblasts, our results showed that cortisol produced in myotubes transactivated GS and suppressed IL-6, which were aborted by RU-486, a GR antagonist, and CBX. Based on the current study, a newly developed cell-based assay using differentiated C2C12 myotubes can be applicable for HTS of inhibitors and exhibits several characteristics as follows. First, it uses a convenient cell line with high levels of endogenous 11β-HSD1 and a simple fluorescence detection technique. In addition, it is useful for the understanding of various biological functions of cortisol in skeletal muscle cells.

Materials and methods

Materials

The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), cortisone, cortisol, and CBX were purchased from Sigma–Aldrich (St. Louis, MO, USA). Human microsomes were purchased from GENTEST (Woburn, MA, USA). Specific primers for IL-6, 11β-HSD1, GR, H6PDH, GS, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed for their amplifications. Enhancer spray, [^{3}H]cortisone, and [^{3}H]cortisol as radioactive substrate and product were purchased from Amersham Life Sciences (Piscataway, NJ, USA). HTRF cortisol assay was purchased from Nihon Schering (Tokyo, Japan). An IL-6 enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals and reagents were purchased from Sigma–Aldrich.

Methods

Cell culture and differentiation of myoblasts into myotubes

C2C12 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Myoblasts were induced to differentiate into myotubes by changing complete medium into differentiation medium containing 2% fetal bovine serum and were allowed to grow further for at least 4 days. To confirm the full differentiation of myoblast into myotube, the morphology of myoblasts and myotubes was observed under phase contrast microscopy using a 10× objective lens and the expression of various markers for myotubes such as myogenin, uncoupling protein-2, and creatine phosphate kinase was examined by reverse transcription polymerase chain reaction (RT–PCR).

11β-HSD1 mRNA expression and assay for enzyme activities

RNAs extracted from myoblasts and C2C12 cells undergoing myogenesis were amplified by RT–PCR using specific primers described below for GR, H6PDH, and 11β-HSD1. GR: 5′-TGC TAT GCT TTG CTC CTG ATC TG-3′, 3′-TGT CAG TTG ATA AAA CCG CTG CC-5′, 62 °C, 30 cycles, 298 bp; H6PDH: 5′-TCG CTG TGA TAG ACT CAA GG-3′, 3′-GGC AGC TGC TGT TGA TCT TA-5′, 55 °C, 25 cycles, 200 bp; 11β-HSD1: 5′-ATG ACC CAG CCT ATG ATT GC-3′, 3′-CTA GTT ACT TAC AAA CAT GTC CTT-5′, 55 °C, 25 cycles, 530 bp. GAPDH was used as an internal standard, and its specific primers are as follows: 5′-TGT TCC TAC CCC CAA TGT GT-3′, 3′-TGT GAG GGA GAT GCT CAG TG-5′, 62 °C, 25 cycles, 200 bp. Aliquots of the PCR reaction were analyzed on 1.5% agarose gel, followed by visualization using ethidium bromide (EtBr) staining, and gel pictures were taken by ultraviolet (UV) transillumination.

To assay microsomal 11β-HSD1 activity, 10 μg human microsome was added in an assay buffer (100 μl) containing 250 μM NADPH, 160 nM cortisone, 20 mM Tris–HCl, and 5 mM ethylenediaminetetraacetic acid (EDTA, pH 6.0) with or without CBX (in dimethyl sulfoxide [DMSO], final 1%) and allowed to incubate for 3 h at 37 °C. Small aliquots (2 μl) of the reaction mixtures were removed and subjected to HTRF assay according to the manufacturer's instructions. HTRF assay is a monoclonal antibody-based competitive assay, and the assay principle of HTRF is schemed later in Fig. 2. The assay is based on a competition between free cortisol and XL665-conjugated cortisol for the binding to an anticortisol antibody labeled with europium (Eu^{3+}) cryptate. Eu^{3+} cryptate and XL665 act as donor and acceptor, respectively. If the two fluorophores will be in close proximity, fluorescence resonance energy transfer (FRET) occurs on excitation. Specific signal is expressed as percentage of Delta F, which is a value calculated from the ratio of 665 nm/615 nm $[(R_{sample} - R_{negative})/R_{negative} \times 100]$, and is inversely proportional to the concentration of cortisol in the sample or the calibrator (see inset in Fig. 2). Cortisol concentration was calculated from the calibration curve obtained from Delta F versus standard solution. IC_{50} values of CBX were determined from concentration-dependent inhibition curves by GraphPad software.

Cellular 11β-HSD1 enzyme activity was measured by conversion of radioactive cortisone into cortisol on thin-layer chromatography (TLC) plates [10]. [^{3}H]Cortisone (160 nM, specific activity 42.0 Ci/mmol) was added to C2C12 culture (500 μl) grown at different cell densities in 24-well plates and incubated for given times. Steroids in culture medium (50 μl) were extracted with ethylacetate (150 μl) and separated by TLC in development solution of chloroform/methanol (9:1, v/v) with visualization of autoradiography of plates sprayed with enhancer on film. On the other hand, a nonradioactive assay was carried out by HTRF protocol. In brief, cells seeded onto 24-well plates at different densities were incubated in DMEM containing 160 nM cortisone (total 500 μl) in the presence or absence of CBX. Samples of the medium (2 μl) were taken at the indicated time, and enzyme activity was assessed by the HTRF method.

Glucocorticoid-dependent transactivation and transrepression

Terminally differentiated C2C12 cells were plated in 6-well plates at a density of 5×10^5 cells/well (final volume 1 ml). On the following day, varying concentrations of cortisone (in DMSO, final 1%) were added to the wells in the presence or absence of either CBX or RU-486 (10 μM in DMSO) and kept in a CO_2 incubator (37 °C, 5% CO_2) for 6 h. Samples of the medium (50 μl) were taken to measure the cortisol level, and then RNAs were extracted with EasyBlue reagent (iNtRON, Songnam, South Korea) according to the manufacturer's instructions, followed by amplification of IL-6 and GS by RT–PCR. The primer sequences for GS and IL-6 are as follows: GS: 5′-GCG AAG ACT TTG GGG TGA TA-3′, 3′-ACT GGT GCC TCT TGC TCA GT-5′, 55 °C, 25 cycles, 162 bp; IL-6: 5′-AGT TGC CTT CTT GGG ACT GA-3′, 3′-TCC ACG ATT TCC CAG AGA AC-5′, 55 °C, 25 cycles, 160 bp. To determine the secreted level of IL-6 in the culture medium by ELISA, myotubes at 1×10^5 cells/well were incubated for 6 h in culture medium containing various concentrations of cortisone. Samples of the medium (50 μl) were taken