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Activation of unfolded protein response pathway is important for valproic acid mediated increase in immunoglobulin G productivity in recombinant Chinese hamster ovary cells

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Process engineering to improve product quality and titers is gaining importance at late-stage cell culture process development. Valproic acid, a US Food and Drug Administration-approved histone deacetylase (HDAC) inhibitor, has been shown to improve cell culture performance with higher productivities and minimal effect on the product quality. However, the wider physiological impact of valproic acid on recombinant cells has not been investigated till date. In this study, we investigate the role of unfolded protein response pathway when immunoglobulin G (IgG)-secreting Chinese hamster ovary (CHO) cells are treated with valproic acid, resulting in a 3-fold increase in product titers and productivity. It is found that cells undergo an early transient endoplasmic reticulum (ER) stress on treatment with valproic acid, and subsequently adapt to perform as high producers. Induction of chaperones through enhanced XBP1 splicing activity and ATF6 activation suggests an increase in protein processing activity in these cells. We show that in addition to the enhanced recombinant mRNA expression of IgG heavy chain and light chain, the activation of unfolded protein response (UPR) pathway is critical to the increase in productivity of cells on valproic acid treatment. Further, upregulation of the UPR pathway is not through HDAC inhibition alone. To our knowledge, this is the first attempt to arrive at a phenotype-genotype mechanistic understanding of how valproic acid treatment enhances productivity in recombinant CHO cells.

[**Key words:** Recombinant Chinese hamster ovary cells; Monoclonal antibody; Valproic acid; Endoplasmic reticulum stress; Unfolded protein response pathway]

Stable recombinant Chinese hamster ovary (CHO) cell lines are the most preferred work horses for the commercial production of biologics such as monoclonal antibodies (1). Enhancing productivity of these cells remains an important objective of any biopharmaceutical industry. One of the strategies to improve product titers at late-stage cell culture process development is by supplementing the culture with small molecule inducers such as sodium butyrate (NaBur) and valproic acid (2,3). While the mode of action of both the additives is through uninterrupted transcription by DNA remodelling and histone deacetylase (HDAC) inhibition (4), valproic acid or 2-propylpentanoic acid is reported to have a lesser impact on culture viability as compared to other HDAC inhibitors (2,5).

Many studies have explored the use of valproic acid (VPA) to increase productivity in recombinant CHO cultures. Treatment of transient CHO and HEK293 cell lines with VPA led to an increase in immunoglobulin G (IgG) titres by 1.5-fold and 5-fold respectively (2). In another study, a 10-fold increase in IgG mRNA is reported in VPA-treated transient CHO cell lines leading to an increase in productivity (6). Addition of higher concentration of VPA at later stages of a fed batch culture also increased product titres with no significant change in the quality of the secreted protein in recombinant CHO cells (7). However, in all of the above studies, efforts to explain the effect of VPA on increasing productivity have been restricted to higher mRNA levels of IgG heavy chain (HC) and light chain (LC).

In addition to inhibition of HDAC-1 and HDAC-2 activities, VPA has been suggested to participate in many different pathways (8–11). It is a US Food and Drug Administration-approved drug used to treat epilepsy and a number of psychological disorders such as bipolar disorder and depression (12). Treatment with VPA has been shown to upregulate endoplasmic reticulum (ER) chaperones, including GRP78 (13) and anti-apoptotic genes, such as BCL-2 (14), while suppressing the expression of CHOP, a pro-apoptotic protein (15). Overall, VPA is proposed to exhibit a protective role, by preventing ER-stress induced damage, in neuronal cell-lines and animal models, such as after spinal cord injury (15) or after retina isochemia (16). The chaperones and the associated unfolded protein response (UPR) signalling pathway genes are also associated with high productivity of recombinant proteins in CHO cells (17,18) and over-expression of various chaperones and transcription factors such as XBP1s (19,20) and ATF6 (21) have resulted in increased titers.

In this work, we determine whether addition of VPA, a potential inducer of recombinant IgG mRNA expression, affects the ER chaperones and the UPR pathway in recombinant cells. We also investigate whether this induction is essential to support the

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increase in IgG titers and productivity in these recombinant CHO cells. To this end, a stable recombinant CHO cell-line secreting anti-rhesus D IgG is treated with varying concentrations of VPA. Based on the growth and final IgG titers, an optimal concentration of VPA is identified to profile the dynamic expression of various ER chaperones and UPR transcription factors in a batch culture. Further, through analogues and UPR inhibitors, we also explore the mechanisms by which VPA increases productivity in stable CHO cells.

MATERIALS AND METHODS

Cell culture Recombinant CHO cell lines secreting anti-rhesus IgG are cultured in a media containing 50% PF-CHO (Thermo-Hyclone) and 50% CD CHO (Gibco-Invitrogen) as described earlier (18).

Cells are maintained at 37°C with 8% CO_2 overlay in Erlenmeyer flasks (Corning, USA). Screening experiments are run in 20 ml batch cultures while the profiling studies are run in duplicates at 300 ml working volume. Adherent CHO-K1 cells are cultured in 6 well plates using Dulbecco's modified Eagle's F12 media (HiMedia) supplemented with 10% fetal bovine serum (Himedia), 2.5 mM L-glutamine (Himedia) and 0.1% antibiotic (Himedia). Viable cell density and culture viability are determined using the tryphan blue dye exclusion method by hemocytometer.

Valproic acid, sodium butyrate, tunicamycin and 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride treatment Filter sterilized 0.1 M stock solution of sodium valproate (Sigma–Aldrich) and sodium butyrate (Himedia) are prepared using Milli Q water. Different concentrations of VPA and sodium butyrate are added to the cultures in duplicates on day 1 with appropriate controls. For short term studies, samples are drawn every 24 h till day 3 while for batch cultures, samples are collected over a period of 9 days to assess the growth, viability and antibody titers and to isolate RNA for gene expression studies. For mock experiments using analogues and UPR inhibitors, cells are treated with 0.5 mM valpromide (Sigma–Aldrich), 2.5 μg/ml of tunicamycin (Sigma–Aldrich) and 125 μM of 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF) (Sigma–Aldrich) on day 1 and samples are collected on day 2.

siRNA treatment The siRNA probe sequences against XBP1s have been reported earlier (22), and are presented in Table S1. The transfection mixture is prepared as follows: 200 pmoles of XBP1s siRNA is diluted with 250 μl of CD-CHO media (Gibco, Thermofisher) and mixed gently. Simultaneously, 10 μl of Lipofectamine 2000 (Thermofisher) is separately diluted with 250 μl of CD-CHO media. Both the reaction mixtures are incubated at room temperature for 5 min. The diluted oligomer is added to the lipofectamine reaction mixture and incubated for 15 min at room temperature to form the oligomer–Lipofectamine 2000 complex. In each well of the culture, 500 μl of this complex is added. The transfection efficiency is assayed using qRT-PCR of XBP1s mRNA post 24, 48 and 72 h of transfection (data shown only for 24 h). The concentration of XBP1s siRNA is optimized to maximize transfection efficiency.

The siRNA treatment experiments are carried out in 6-well plates. Briefly, Recombinant CHO cells are cultured in Erlenmeyer flasks (Corning, USA) at a seeding density of 3×10^5 cells/mL in a total culture volume of 50 ml. On day 1, 2.5 ml of the culture is aseptically transferred to each well of a 6 well plate, and treated with siRNA, VPA or both simultaneously. As a negative control, 500 μl of CD-CHO media alone is added to one of the wells.

Quantification of intracellular and secreted antibody Culture supernatants are collected at regular intervals and stored at −20°C for antibody quantification by enzyme-linked immunosorbent assay (ELISA) using the protocol as described earlier (23). Human IgG (Sigma–Aldrich) is used as a standard and the productivities are calculated as described earlier. For intracellular IgG quantification an anti-human IgG (Fc-specific) fluorescein isothiocyanate (FITC) labelled antibody produced in goat (Sigma–Aldrich, Cat. no. F9512) is used. 10 million cells are washed with PBS by spinning down at 3000 RPM for 5 min and fixed by re-suspending the pellet in 2% chloroform. The obtained pellet was permeated using 70% ethanol and labelled with 1:64 diluted IgG-FITC (Sigma). Cells without antibody are used as a negative control to set the gates and identify the cell population. A minimum of 10,000 counts is registered for all samples to arrive at a statistically significant data using BD FACS Aria (BD Biosciences, USA). The data analysis is performed using the Flowjo software (Treestar, USA).

Glucose and lactate quantification The level of glucose in culture supernatant is measured using a GOD-PAP glucose assay kit (Biolab Diagnostics). Briefly, to 10 μl of culture supernatant, 1 ml of assay reagent is added and incubated at 37°C for 5 min. Glucose oxidase present in the assay reagent reacts with glucose and converts amino-4 antipyrine, in the presence of phenol peroxidise and H_2O_2, to quinoeimine, that has an absorption maximum at 500 nM. Lactate is measured using an enzyme assay optimized in-house. Lactate is converted to pyruvate in the presence of lactate dehydrogenase. During this conversion, NAD is converted to NADH which has a maximum absorbance at 340 nm. As conversion of lactate to pyruvate is reversible, hydrazine is added in excess to promote the conversion of pyruvate to pyruvate-hydrazone, an irreversible complex. The entire reaction is carried out in a total volume of 305 μl buffer solution (pH 9.0) containing 0.5 M Glycine (Sigma–Aldrich), 0.4 M Hydrazine (Himedia), 17 mg/ml of NAD (Sigma–Aldrich) and 0.4 U/ml of lactate dehydrogenase (Sigma–Aldrich). Lactic Acid (Sigma) is used as standard and the concentration of lactate in samples are calculated using a standard curve.

Quantitative real time polymerase chain reaction Total RNA is isolated from cultured cells using TRI reagent (Sigma–Aldrich) as per the manufacturer's instruction and quantified using Nanodrop spectrophotometer (Implen, Germany). Total RNA (4 μg) is converted to cDNA in a total volume of 20 μl using a Revert Aid H Minus M-MuLV Reverse Transcriptase Kit (Thermo Scientific, Canada) following the manufacturer's instruction. Using the QuantiFast SYBR Green PCR kit (Qiagen, USA) and iQ Bio-rad Sybr green mix (Bio-Rad), 100 ng of cDNA is used for quantitative real time PCR in a total volume of 25 μl supplemented with 0.5 μM gene specific primers in duplicates (Table S1). β-Actin is used as the house keeping gene and the expression levels are computed using the $\Delta\Delta C_T$ method by normalization with the C_T values of another cell line which had the lowest expression profiles of all the UPR genes. Detailed description of the calculations have been described earlier (18).

Cell cycle analysis Ten million cells are washed with sterile 1X PBS and fixed by incubating with 75% ethanol for 1 h in ice. Post incubation, cells are washed briefly with PBS thrice and suspended in 0.5 ml of PBS containing 0.25% of Triton X (Sigma–Aldrich). Cells are then incubated for 15 min and washed with PBS thrice. Post centrifugation, cell pellets are re-suspended in 0.5 ml of propidium iodide (PI) solution containing 10 μg/ml of RNase (Thermo Scientific) and 20 μg/ml of PI (Sigma–Aldrich). Cells are sorted using FACS-ARIA (BD Biosciences, USA) and a minimum of 10,000 counts are recorded to obtain statistically significant data. The obtained data is further analysed using the Flowjo software (Treestar, USA) for cell cycle analysis and intracellular IgG quantification.

Data analysis All data is expressed as mean ± standard deviation (SD) from two biological replicate cultures, with atleast two technical replicates from each culture. Student's *t*-test was employed to estimate statistical significance.

RESULTS AND DISCUSSION

Shift in growth and metabolism of CHO cells under VPA treatment CHO cells are treated with varying concentrations of VPA in a batch culture, to identify an optimal concentration to be used for further studies. A concentration dependant decrease in maximum cell density and viability is seen in VPA-treated cultures with 0.5 mM treated cells exhibiting least inhibition on growth along with maximum IgG titers. Hence, this concentration is chosen for further profiling in a 300 ml batch culture. Note that HDAC inhibition is also reported to be effective at millimolar concentrations of VPA within the therapeutic range (0.35 mM–0.7 mM) (24).

Fig. 1A illustrates the effect of VPA (0.5 mM) treatment on growth and viability of recombinant CHO cells. Treated cells reached a maximum viable cell density of 4.4 million cells/ml on day 4, while control cultures reach upto 6 million cells/ml on the same day. Although the growth rate of treated cells is lower than untreated cells, high viability is maintained for a longer period, reflected by the lower cell death rate of treated cells. The decrease in growth rate is accompanied by a shift in metabolism where cells treated with VPA produce less lactate per mole of glucose consumed (Fig. 1B). Note that cumulative glucose consumption rates (q_S) are similar between control and VPA-treated cells, while lactate production rate (q_L) is lower in treated cells from day 6 onwards (data not shown). VPA also impacts the immediate distribution of cells in various cell cycle phases. More than 60% of cells on day 2 are in G1-phase in VPA-treated cells (Fig. 1C) compared to around 40% in untreated cells. Cell cycle arrest at G1 phase has been reported earlier in medulablastoma cell lines treated with VPA (25) and CHO cultures treated with NaBur (26) and valeric acid (pentanoic acid) (27), associated with increased productivities in treated CHO cultures. Enrichment of G1-phase cells potentially induces a shift in cellular metabolism and reprograms the cells to increase recombinant protein productivity (28).

Time profiles of antibody titres, productivities and recombinant mRNA expression VPA treatment increased IgG titers, with final titers of ~1400 μg/ml on day 9 in treated cultures

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