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Metabolism and metabolic burden by α_1-antitrypsin production in human AGE1.HN cells

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ABSTRACT

The metabolic burden on human AGE1.HN cells imposed by the production of recombinant α_1-antitrypsin (A1AT) was studied by comparing a selected high-producing clonal cell line with the parental cell line. RNA, lipid, and phosphatidylcholine fractions were higher in the producer cell line causing metabolic changes in the producer, e.g., increased glycine and glutamate production. By simulating the theoretical metabolite demand for production of mature A1AT using a network model, it was found that the differences in metabolic profiles between producer and parental cells match the observed increased C1-unit and nucleotide demand as well as lipid precursor demand in the producer. Additionally, metabolic flux analysis revealed similar energy metabolism in both cell lines. The increased lipid content seems related to activated secretion machinery in the producer cell line. Increased lipid and C1 metabolism seem important targets for further improvement of AGE1.HN and other producing mammalian cells.

1. Introduction

Alpha 1-antitrypsin (A1AT) deficiency is an inherited disease characterized by low serum levels of A1AT which can result in lung emphysema and liver dysfunction (Kelly et al., 2010; Petrache et al., 2009). The lack of protection against neutrophil elastase usually provided by A1AT leads to destruction of lung parenchyma and finally emphysema in the respiratory tract. The mortality rate of patients can be decreased by A1AT augmentation which is achieved by intravenous infusion of a purified preparation of human A1AT (A1AT-Group, 1998). However, only human plasma-derived products are licensed by the US-FDA for intravenous treatment of A1AT deficiency in spite of many attempts to produce recombinant A1AT in prokaryotic and eukaryotic hosts (Karnaukhova et al., 2006). This fact makes the therapy currently very expensive and not cost-effective (Gildea et al., 2003) showing that an alternative source of A1AT would be important. Recently, it was shown that A1AT derived from the novel human cell line AGE1.HN has similar anti-inflammatory activity as plasma-derived A1AT (Blanchard et al., 2011) indicating that this cell line is a useful expression system for large scale production of this biopharmaceutical. Recent studies on growth, metabolism (Niklas et al., 2011b,c,d,e) and glycosylation properties (Blanchard et al., 2011) of this cell line have shown that AGE1.HN seems a favorable cell line for the production of glycoproteins requiring human-type post-translational modifications. Further improvement of the productivity might be achieved by a detailed understanding of the cellular protein production process.

Selected cellular principles underlying varying productivity were analyzed using different omics-technologies (Alete et al., 2005; Hayduk and Lee, 2005; Lee et al., 2007; Seth et al., 2007; Smales et al., 2004; Yee et al., 2009). Different optimization strategies (Dietmair et al., 2011), based on genetic or metabolic engineering, not only in microorganism (Anthony et al., 2009; Ma et al., 2011; Tsao et al., 2010) but also in mammalian cells (Baik et al., 2012; Ng et al., 2007; Voedisch et al., 2011) were proposed. In proteomic analyses of cells with varying secreted recombinant protein productivities changes in, e.g., translation, energy metabolism, chaperones, and cytoskeletal proteins were observed (Alete et al., 2005; Hayduk and Lee, 2005; Smales et al., 2004). Seth et al. (2007) analyzed changes in transcriptome and proteome in different producers and proposed that differences in protein synthesis and cell growth controlling networks might lead to high productivity. Another transcriptome study concluded that particularly vesicle trafficking, endocytosis and cytoskeletal elements go along with increased specific productivity (Yee et al., 2009). Khoo et al. (2007) compared the transcriptome of producer and non-producer cell lines. They found that the producer cells were not growing slower indicating negligible metabolic burden in this case but nevertheless differences in metabolic activities

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were observed. It was concluded that slower growth as a result of metabolic burden might be negligible in cells that were selected for high productivity as well as growth rate. To our knowledge a detailed analysis on the metabolome level concerning differences in productivity was not described so far.

In this study, we analyzed the metabolic burden of α_1-antitrypsin production in AGE1.HN. The goal was to compare growth, dynamics of biomass constituents, metabolite dynamics, and metabolic fluxes in the A1AT producing cell line AGE1.HN.AAT and its parental cell line. The following questions were addressed: (i) what are the differences in growth and biomass composition of both cell lines, (ii) what is particularly distinguishing this clonal A1AT producing cell line from the parental cell population enabling this cell line to produce large quantities of the product, and (iii) can we identify potential targets for further improvement of the cell line by understanding the metabolic requirements of A1AT production and the adaptation of the cell to high-level glycoprotein secretion?

2. Material and methods

2.1. Cell line

The cell line AGE1.HN® (ProBioGen, Berlin, Germany) originates from primary human brain cells. These were immortalized and improved using different adenoviral genes as described previously (Niklas et al., 2011a). The α_1-antitrypsin (A1AT) producing cell line AGE1.HN.AAT was developed as follows. Parental cells were transfected with an expression vector containing the human A1AT gene driven by a human CMV/EF1 hybrid promoter (ProBioGen). High producing clones were established with puromycin drug selection. In a recent publication, it was shown that A1AT derived from AGE1.HN cells was highly active and showed a complex glycostructure (Blanchard et al., 2011).

2.2. Analytical methods

2.2.1. Cell counting was performed using an automated cell counter (Countess, Invitrogen, Karlsruhe, Germany). Viability was assessed using the Trypan blue exclusion method.

2.2.2. *Cell dry weight*: Cell suspensions containing 3 or 4×10^6 cells were harvested ($50 \times g$, 5 min, 25 °C, Labofuge 400 R Function Line, Heraeus Instruments, Hanau, Germany) and the pellets resuspended in 2 ml 37 °C PBS. The suspensions were transferred into preweighed Eppendorf tubes and again centrifuged ($900 \times g$, 5 min, 20 °C, Picofuge, Heraeus Instruments, Hanau, Germany). The supernatant was discarded. The pellets were frozen at −70 °C, lyophilized, and finally weighed.

2.2.3. *Quantification of intracellular and extracellular proteins.* Cell pellets from the cell dry weight analysis were resuspended in 200 µl CelLytic™ M solution (Sigma-Aldrich, Steinheim, Germany) and incubated for 15 min at 20 °C on a shaker (400 min^{-1}, MS1 Minishaker, IKA, Staufen, Germany). After centrifugation ($9500 \times g$, 10 min, 4 °C, Biofuge fresco, Heraeus Instruments, Hanau, Germany), the supernatants were transferred into new tubes. Proteins were quantified using Bradford assay (Bradford, 1976) (reagent from Bio-Rad Laboratories, Munich, Germany). A series of BSA (bovine serum albumin) solutions was used for calibration. The absorption was measured at 540 nm (iEMS Reader MF, Labsystems, Helsinki, Finland). The total extracellular protein concentration was directly analyzed in culture supernatants using the Bradford assay.

2.2.4. *Extracellular protein analysis*

The culture supernatants were mixed with an equal amount of sample buffer (0.45 M Tris–HCl, pH 6.8; 3.5 ml dH_2O; 1.6 ml 10% glycerol; 0.4 ml β-mercaptoethanol; 0.4 ml bromphenol blue) and incubated for 5 min at 100 °C. After that, the samples were loaded on the gel and analyzed using a 4% stacking gel and a 12.5% running gel (Laemmli, 1970). Subsequently, the gel was stained for 20 min using Coomassie Blue (0.2% Coomassie R250, 0.005% Coomassie G250i, 10% ethanol, 40% methanol, 2% glycerin). The stained gel was scanned and the intensity of the bands was quantified using ImageQuantTM TL (Amersham, Biosciences/GE Healthcare, Little Chalfont, Buckinghamshire, Great Britain). Selected bands were cut out and collected in separate tubes. The gel pieces were washed with 100 µl MilliQ water in a thermo shaker (5 min, 500 1/min, 37 °C; Thermomixer comfort, Eppendorf AG, Hamburg, Germany). The MilliQ water was removed and the washing step was repeated with 100 µl 50% acetonitrile. Subsequently, the acetonitrile was removed and the gel pieces were incubated with 50 µl 10 mM dithiothreitol (DTT) for 1 h at 60 °C. After that, the DTT was removed, the gel pieces washed with 100 µl MilliQ water as described above, and then incubated with 50 µl iodoacetamide (50 mM in MilliQ water) for 30 min at room temperature in the dark. Then, the iodoacetamide was removed and the samples were washed five times with 100 µl 50% acetonitrile as described above. The gel pieces were washed twice with 100 µl acetonitrile and then evaporated to dryness at 37 °C for 10 minutes. 10 µl trypsin solution (0.5 µg/µl in 40 mM NH_4HCO_3) were added and incubated (5 min, 500 1/min, 37 °C) until the solution was absorbed by the gel pieces. Remaining trypsin solution was removed and the gel pieces were covered with 20 µl of 40 mM NH_4HCO_3 and digested over night (500 l/min, 37 °C). To stop the digestion, 0.5 µl 10% trifluoroacetic acid (TFA) were added and incubated for 15 min (500 1/min, 37 °C). The supernatants were transferred into fresh tubes and the gel pieces were extracted once more with 20 µl 0.1% TFA (30 min, 1000 1/min, 37 °C). The supernatants were pooled and a third extraction step with 20 µl 50% acetonitrile (30 min, 1000 1/min, 37 °C) was performed and the supernatants pooled again. In the end, the supernatants were concentrated to a volume of 10 µl. For the identification of the excised proteins, 2.5 µl of the supernatants were mixed with 2.5 µl α-cyano-4-hydroxycinnamic acid (5 mg/ml in 70% acetonitrile). Per spot, 1 µl of the sample-matrix-mixture was pipetted on the MALDI-target. After crystallization, the samples were analyzed using a MALDI-ToF/ToF 4800 mass spectrometer (Applied Biosystems, Darmstadt, Germany). An external calibration was performed prior to the measurement using standard peptides (4700 calibration mixture, Applied Biosystems, Darmstadt, Germany). Spectra were recorded in positive ion mode with reflector in the range between 800 and 4000 m/z. The six most abundant fragments were selected for MS/MS analysis. MS/MS analysis was carried out in 1 kV positive ion mode with air as collision gas. The recorded spectra were searched against the SwissProt database (v2010/6) using the MASCOT v2.1 search engine implemented in the GPS ExplorerTM (Applied Biosystems, Darmstadt, Germany). The maximum number of missed cleaving sites for the selected digestion enzyme trypsin was 1. Allowed modifications were methionine oxidation and cysteine carbamidomethylation. Mass tolerance was set to 50 ppm during the analysis of the MS spectra and to 0.3 Da during the analysis of the MS/MS spectra.

2.2.5. *RNA content* was determined using the TRI Reagent® Solution from Applied Biosystems/Ambion (Austin, TX, USA) according to the manual. The RNA concentrations were determined spectrophotometrically (Genesys 10 Bio, Thermo Fisher Scientific, Waltham, MA, USA).

Total lipid analysis. Cells were cultured in 250 ml shake flasks (Corning) and harvested in the exponential growth phase by