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Metabolic flux rearrangement in the amino acid metabolism reduces ammonia stress in the α_1-antitrypsin producing human AGE1.HN cell line

Christian Priesnitz [a,1], Jens Niklas [a,1], Thomas Rose [b], Volker Sandig [b], Elmar Heinzle [a,*]

[a] Biochemical Engineering Institute, Saarland University, D-66123 Saarbrücken, Germany
[b] ProBioGen AG, D-13086 Berlin, Germany

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ABSTRACT

This study focused on metabolic changes in the neuronal human cell line AGE1.HN upon increased ammonia stress. Batch cultivations of α_1-antitrypsin (A1AT) producing AGE1.HN cells were carried out in media with initial ammonia concentrations ranging from 0 mM to 5 mM. Growth, A1AT production, metabolite dynamics and finally metabolic fluxes calculated by metabolite balancing were compared. Growth and A1AT production decreased with increasing ammonia concentration. The maximum A1AT concentration decreased from 0.63 g/l to 0.51 g/l. Central energy metabolism remained relatively unaffected exhibiting only slightly increased glycolytic flux at high initial ammonia concentration in the medium. However, the amino acid metabolism was significantly changed. Fluxes through transaminases involved in amino acid degradation were reduced concurrently with a reduced uptake of amino acids. On the other hand fluxes through transaminases working in the direction of amino acid synthesis, i.e., alanine and phosphoserine, were increased leading to increased storage of excess nitrogen in extracellular alanine and serine. Glutamate dehydrogenase flux was reversed increasingly fixing free ammonia with increasing ammonia concentration. Urea production additionally observed was associated with arginine uptake by the cells and did not increase at high ammonia stress. It was therefore not used as nitrogen sink to remove excess ammonia. The results indicate that the AGE1.HN cell line can adapt to ammonia concentrations usually present during the cultivation process to a large extent by changing metabolism but with slightly reduced A1AT production and growth.

1. Introduction

In the last decades, mammalian cell cultures gained more and more importance in biotechnological and pharmaceutical industries. Apart from the use of mammalian cells in the prediction of toxicity (Noor et al., 2009), they became more and more popular for the production of biopharmaceuticals and especially therapeutic proteins (Wurm, 2004). This was mainly due to their ability of posttranscriptional modification of the expressed proteins. By now, the number of therapeutic proteins produced in mammalian cells exceeds the number produced in bacteria (Walsh, 2010). The most important posttranscriptional modifications are N- and O-linked glycosylation. Glycosylation plays an important role for protein stability, ligand binding, immunogenicity, and serum half-life (Walsh, 2006). Much effort was put into optimizing productivity and product quality by applying different strategies like engineering cell metabolism, protein secretion, apoptosis resistance, and glycosylation (Lim et al., 2010; Zhang et al., 2010). One major factor that still limits productivity is the accumulation of toxic byproducts like lactate and ammonia in the cell culture medium. Ammonia results on the one hand from chemical decomposition of glutamine (Tritsch and Moore, 1962), which is an important component in most cell culture media, and on the other hand from metabolic deamination reactions. The effects of increased ammonia concentrations on different mammalian cells were examined by several groups (Mirabet et al., 1997; Ozturk et al., 1992; Schneider et al., 1996). The large variation of the influence of ammonia on different cells described in the literature shows that it is important to analyze the effects of elevated ammonia levels for every cell line separately and that it is not possible to completely transfer results obtained for one cell line to another. The effects range from decreased cell growth and reduced productivity to alterations in the glycosylation pattern and inhibition of virus multiplication (Andersen and Goochee, 1995; Borys et al., 1994; Chen and Harcum, 2006; Hong et al., 2010; Koyama and Uchida, 1989; Thorens and Vassalli, 1986; Yang and Butler, 2000, 2002) and are at least partly attributed to changes in the intracellular pH and changes in UDP-hexose levels (Ryll et al., 1994). To overcome these negative effects of ammonia on cell growth, productivity, and

* Corresponding author. Fax: +49 681 302 4572.
E-mail address: e.heinzle@mx.uni-saarland.de (E. Heinzle).
[1] Authors contributed equally

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product quality the reduction of the ammonia accumulation in the media is a major goal. To achieve this, a variety of different approaches were proposed. They include (over)expression of the glutamine synthetase (Bell et al., 1995; Cockett et al., 1990), controlled feeding of glutamine (Eyer et al., 1995; Glacken et al., 1986), feeding of glutamine according to the demand for biosynthesis (Xie and Wang, 1994) or depending on the oxygen uptake rate (Eyer et al., 1995), co-culturing CHO cells with HepG2 cells (Choi et al., 2000), substitution of glutamine by glutamate, asparagine (Kurano et al., 1990) or α-ketoglutarate (Hassell and Butler, 1990), and adaptation of cells to high ammonia concentrations (Schumpp and Schlaeger, 1992). Henry et al. described reduced ammonia formation also upon expression of pyruvate carboxylase in HEK-293 cells (Henry and Durocher, 2011). Most of the investigations available deal with the effects of elevated ammonia levels in hybridoma and myeloma cells used for the production of monoclonal antibodies (McQueen and Bailey, 1990; Miller et al., 1988; Ozturk et al., 1992; Reuveny et al., 1986) and comparatively few publications can be found about the effects in CHO cells (Yang and Butler, 2000), BHK cells (Cruz et al., 2000) or other mammalian cell lines although they are among the most prominent cell lines for production of therapeutic proteins. In most publications, the investigated parameters were just cell growth and productivity and only in some studies changes in extracellular metabolites were additionally analyzed. Detailed studies on the effects of elevated ammonia concentrations on central metabolism and intracellular fluxes are hardly available (Bonarius et al., 1998; Nadeau et al., 2000). Metabolome analysis (Chrysanthopoulos et al., 2010; Hiller et al., 2011) and especially metabolic flux analysis (MFA) (Stephanopoulos, 1999) represent powerful tools to investigate and analyze the effects of different conditions on the metabolism of cells (Ahn and Antoniewicz, 2011; Maier et al., 2009). In mammalian cells, MFA was applied for a variety of cell lines and in a variety of fields (Niklas and Heinzle, 2011) such as toxicology (Niklas et al., 2009; Strigun et al., 2011a), medical research (Gaglio et al., 2011; Lee et al., 2003; Strigun et al., 2011b), and biopharmaceutical production (Boghigian et al., 2010; Bonarius et al., 2001; Khoo and Al-Rubeai, 2009; Niklas et al., 2011a, 2011b, 2011c; Zupke and Stephanopoulos, 1995). The main goal of the presented study was a detailed analysis of the effects of elevated ammonia concentrations on growth, metabolism, and glycoprotein production in the human cell line AGE1.HN (ProBioGen, Berlin, Germany) that was specifically designed for production of biopharmaceuticals requiring complex human-type glycosylation and viral vaccines (Blanchard et al., 2011). In this study an AGE1.HN cell was used that is producing α_1-antitrypsin (A1AT). This glycoprotein, which is produced in vivo in the human liver, has three *N*-glycosylation sites and requires complex glycosylation (Carrell et al., 1982; Kolarich et al., 2006). A1AT is an important biopharmaceutical that is required for augmentation therapy in A1AT deficiency, a hereditary disorder which may result in a shortened lifetime mainly caused by chronic respiratory insufficiency (Tonelli and Brantly, 2010). So far, only plasma-derived human A1AT is approved for augmentation therapy whereas the production of stable and active recombinant or transgenic A1AT in several non-human hosts was impeded by impurities or lower stability mainly caused by wrong glycosylation of the produced A1AT compared to the plasma-derived version (Karnaukhova et al., 2006).

In this study the following questions were addressed: (i), what is the effect of ammonia on cell growth, A1AT formation, A1AT quality/activity, (ii), how does the metabolism of AGE1.HN change upon high ammonia supply and how is ammonia detoxified, and (iii), should further engineering focus on ammonia metabolism? The results are important for further rational improvement of human production cell lines based on a thorough understanding of cellular physiology.

2. Material and methods

2.1. Cell line

The AGE1.HN® cell line (ProBioGen AG, Berlin, Germany) was derived from a primary human tissue sample from the periventricular zone of a fetal brain. The cells were immortalized using an expression plasmid which contained the adenoviral genes E1 A and B of the human Adenovirus 5. These genes were driven by the human pGK and the endogenous E1B promoter, respectively. Furthermore, the cell line was improved by expressing the protein pIX of the human Adenovirus 5. This expression leads to changes in the metabolism, enhanced productivity of secreted proteins, and increases the susceptibility to various viruses. This AGE1.HN cell line expresses marker genes for neuronal cells but lacks expression of glial marker proteins. The cell line was transfected with the expression vector containing human A1AT (ProBioGen AG) under the control of a specific CMV/EF1 hybrid promoter (ProBioGen AG). High producer cells were selected with puromycin. It was shown recently that this cell line can produce fully active A1AT with its complex glycosylation pattern (Blanchard et al., 2011).

2.2. Cell culture and experimental procedure

The cells were cultured in shake flasks (Corning, NY, USA) or 50 ml filter-tube bioreactors (TPP, Trasadingen, Switzerland) at 37 °C in a 5% CO_2 supplied shake-incubator (185 1/min, 2 min. shaking orbit, Innova 4230, New Brunswick Scientific, Edison, NJ, USA). The pre-culture was carried out in a 250 ml shake flask in serum-free 42-Max-UB-medium (Teutocell AG, Bielefeld, Germany). The cells were centrifuged (500 1/min, 5 min, 22 °C, Labofuge, Heraeus Instruments, Hanau, Germany) and the supernatant discarded. The pellet was resuspended in 30 ml PBS (37 °C, PAA Laboratories, Pasching, Austria) and centrifuged again. After discarding the supernatant the pellet was resuspended in 42-Max-UB-medium supplemented with 2 mM glutamine. Three 50 ml filter-tube bioreactors were inoculated yielding an initial cell density of about 9×10^5 cells/ml. Through the addition of PBS or PBS containing NH_4Cl (125 mM and 250 mM), ammonium concentrations of 0 mM, 2.5 mM, and 5 mM were achieved. The final culture volume was 18 ml. Samples (300 μl) were taken every day. 30 μl were used for cell counting. The rest was centrifuged (10,000 1/min, 5 min, 22 °C, Biofuge pico, Heraeus Instruments, Hanau, Germany) and the supernatant transferred into fresh tubes. Of the samples, 70 μl were used for pH determination (MP 220 pH-Meter, Mettler-Toledo, Giessen, Germany), 20 μl for ammonia measurements, and the rest was frozen (−20 °C). The analysis of cultivation parameters was carried out using an automated cell counter (Countess, Invitrogen, Karlsruhe, Germany) which determines cell density, viability (Trypan Blue exclusion method), and cell size.

2.3. Quantification of metabolites

Glucose, lactate, and pyruvate in the supernatant were analyzed using high pressure liquid chromatography (HPLC) as described previously (Niklas et al., 2009). Quantification of proteinogenic amino acids was performed by another HPLC-method (Kromer et al., 2005). Glutamine data was corrected for degradation as described recently (Niklas et al., 2011c).

2.4. Quantification of ammonia and α_1-antitrypsin

Ammonia was quantified using an ammonia assay kit (Sigma–Aldrich, Steinheim, Germany) according to the instructions. The sample volume was 20 μl and the absorption was measured in a spectrophotometer at 414 nm (iEMS Reader MF, Labsystems,