Glycosylation of CHO-Derived Recombinant tPA Produced under Elevated pCO_2

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Carbon dioxide is a metabolic byproduct of mammalian cell metabolism that can accumulate in poorly ventilated cultures. A buildup of CO_2 at constant pH will be accompanied by an increase in medium osmolality. We have examined the glycosylation of tissue plasminogen activator (tPA) produced under serum-free conditions by recombinant Chinese hamster ovary (CHO) cells (MT2-1-8 cell line) in response to elevated pCO_2 at constant or elevated osmolality. The proportion of sialic acids comprising *N*-glycolylneuraminic acid decreased from 2.3−4.0% under 36 mmHg pCO_2 to 1.5−2.2% under 250 mmHg pCO_2. No changes were observed in the total sialic acid content, the content of other monosaccharides, the relative amounts of type I and type II tPAs, the distribution of surface charges, or the proportion of high-mannose oligosaccharides—even though these conditions have previously been shown to inhibit the specific growth rate of MT2-1-8 cells by 30−40% and the specific tPA production rate by as much as 40%. These results suggest robust glycosylation of tPA by CHO cells.

Introduction

Accumulation of CO_2 has recently been recognized as a limiting factor in mammalian cell cultures by several industrial groups (Aunins and Henzler, 1993; Ozturk et al., 1995; Gray and Chen, 1996). Increased CO_2 partial pressure (pCO_2) at constant pH results in increased osmolality due to the associated buildup of bicarbonate ions in the medium. We have shown that exposure to elevated pCO_2 at constant or elevated osmolality can inhibit the specific growth and tPA production rates of a recombinant Chinese hamster ovary (CHO) cell line (Kimura and Miller, 1996).

High pCO_2 levels are likely to affect intracellular pH (pH_i), even if the medium pH is controlled. Carbon dioxide is a nonpolar molecule that readily diffuses across the cell membrane, where it can hydrate and then dissociate into H^+ and HCO_3^- (Alberts et al., 1989). Mammalian cells exposed to high pCO_2 at constant pH experience an initial decrease in pH_i (Krapf et al., 1988). The increased activity of Na^+/H^+ and/or HCO_3^-/Cl^- antiporters will counteract this effect, but pH_i may or may not return to the original value (Krapf et al., 1988; Simchowitz and Roos, 1985). Changes in pH_i can affect the activity of cytosolic enzymes or alter the metabolism of cells (Madshus, 1988). High pCO_2 levels may also alter the pH in the endoplasmic reticulum and/or the Golgi apparatus. Changes in the pH of these organelles could alter protein processing (including glycosylation; Thorens and Vassalli, 1986) and secretion.

Glycosylation can influence the activity, clearance rate, antigenicity, immunogenicity, and solubility of proteins (Goochee et al., 1992; Jenkins and Curling, 1994). Thus, glycosylation microheterogeneity is an important issue in the production of protein therapeutics. Grampp et al. (1994) reported that the amount of *N*-glycolylneuraminic acid (relative to *N*-acetylneuraminic acid) present in recombinant glycoproteins expressed by CHO cell lines varied with pCO_2 in the range from 10 to 160 mmHg. However, we are not aware of any other reports on the influence of elevated pCO_2 on protein glycosylation in mammalian cell culture.

Previously, we reported a dose-dependent inhibition of cell growth by elevated pCO_2 in the range 36−250 mmHg; 250 mmHg pCO_2 inhibited the specific growth rate of the MT2-1-8 CHO cell line by 30% under constant osmolality and by 40% under elevated osmolality conditions (Kimura and Miller, 1996). The specific tPA production rate (q_{tPA}) was inhibited by 40% at 250 mmHg at constant osmolality. Although q_{tPA} was inhibited by 25% at 140 mmHg and elevated osmolality (342 mOsmol/kg), it was not significantly affected by 250 mmHg and elevated osmolality (376 mOsmol/kg). Here, we examine the effects on tPA glycosylation due to 250 mmHg pCO_2 at both 310 and 376 mOsmol/kg.

CHO-cell-derived tPA has three N-linked glycosylation sites, one of which (Asn-117) is occupied primarily by high-mannose oligosaccharides with a small amount of hybrid oligosaccharides (Guzzetta et al., 1993) and the other two (Asn-184 and -448) by complex oligosaccharides (Spellman et al., 1989). TPA also contains an O-linked fucose at Thr-61 (Harris et al., 1991). The Asn-184 site is variably glycosylated, resulting in two tPA glycoforms with three (type I) and two (type II) oligosaccharides, respectively. Variable glycosylation at Asn-184 affects both the *in vitro* activity and the clearance rate of tPA (Wittwer et al., 1989; Cole et al., 1993). Increased sialylation of the complex oligosaccharides or removal of the high-mannose oligosaccharides has been shown to decrease the clearance rate (Hotchkiss et al., 1988; Cole et al., 1993). TPA can be cleaved at the Arg-275 site to form two-chain tPA (vs one-chain tPA). Two-chain tPA has similar enzymatic activity but is more susceptible to protease inhibitors *in vivo* (de Serrano and Castellino, 1990).

TPA produced under serum-free conditions was analyzed for changes in glycosylation by monosaccharide analysis. However, it should be noted that some changes

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could occur in the oligosaccharide structure without being detectable by monosaccharide analysis. Only a complete oligosaccharide profiling technique can unambiguously confirm such changes. In light of this fact, we also performed assays to examine the oligosaccharide structures of tPA that are important for its *in vivo* activity. TPA was analyzed by SDS-PAGE (to distinguish between type I vs type II tPA and one-chain vs two-chain), lectin blotting (specific for high-mannose oligosaccharides), and isoelectric focusing (primarily sialic acid distribution).

Materials and Methods

Cell Line and Medium. The MT2-1-8 cell line (obtained from SmithKline Beecham Pharmaceuticals, King of Prussia, PA) was created by transfecting a $DHFR^-$ (dihydrofolate reductase negative) CHO cell line (Urlaub and Chasin, 1980) with a vector containing the DHFR and human tPA genes. MT2-1-8 cells were grown in a custom low-salt (35.5 mg/L) DMEM (Dulbecco's Modified Eagle's medium):Ham's F12 mixture with 15 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid] (Irvine Scientific; Santa Ana, CA), which was supplemented with MEM (minimum essential medium) nonessential amino acids (diluted to 1×; Gibco, Grand Island, NY), sodium bicarbonate (29 mM), glutamine (to 6 mM; Gibco), HEPES (to 25 mM), sodium selenite (20 nM), ethanolamine (20 μM), methotrexate (200 nM), bovine serum albumin (BSA; 250 mg/L), bovine insulin (1.25 mg/L), bovine holo-transferrin (7.5 mg/L), and 3.5% (v/v) FBS (fetal bovine serum; Gibco). All medium additives and reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Milli-Q water (18 MΩ resistance; Millipore, Bedford Park, MA) was used for all experiments.

Medium used for stock cultures, control experiments at 36 mmHg pCO_2 (5% CO_2), and high-pCO_2 experiments not corrected for osmolality was supplemented with 5.71 g/L of NaCl (36 mmHg medium). Medium for experiments at 140 and 250 mmHg pCO_2 conducted at an osmolality of 310 mOsmol/kg were supplemented with 4.73 (140 mmHg−310 mOsmol/kg of medium) and 3.62 g/L (250 mmHg−310 mOsmol/kg of medium) of NaCl, respectively.

High pCO_2 Experiments. MT2-1-8 cells were seeded into T-150 flasks at a density of 10^6 cells per T-flask in 50 mL of 36 mmHg medium and placed in a 5% CO_2 incubator. The flasks were grown to 50% confluence, at which point the medium was aspirated out of the T-flasks and replaced with 50 mL of 36 mmHg medium without FBS (serum-free medium) to remove any serum proteases adsorbed to plastic surfaces or cells. After 5−6 h, the 36 mmHg serum-free medium was replaced with the appropriate pre-equilibrated serum-free medium. The T-flasks were then placed in a 5% CO_2 incubator or a modular incubator chamber gassed with the appropriate CO_2/air mixture (Kimura and Miller, 1996). After 2 days, the medium was removed from the T-flasks and frozen for later analysis. Independent experiments for each set of culture conditions (pCO_2 and osmolality values) are indicated by Roman numerals (I, II, III).

Purification of tPA. An *Erythrina* trypsin inhibitor (ETI; American Diagnostica, Greenwich, CT) column was used to purify tPA from serum-free cell culture medium (Heussen et al., 1984). ETI (10 mg) was attached to 2 mL of Affi-prep 10 high-pressure chromatographic support (Bio-Rad, Hercules, CA) according to the instructions from Bio-Rad.

Cell culture supernatant (100 mL) was centrifuged, concentrated to approximately 40 mL in Centriprep-10 centrifugal concentrators (Amicon, Beverly, MA), and then adjusted to 0.01% (w/v) Triton X-100. After the concentrated supernatant was loaded onto the column, the column was washed with low-salt buffer (50 mM sodium phosphate, 100 mM NaCl, 0.01% (w/v) Triton X-100, pH 7.2) until the absorbance at 280 nm of the column effluent returned to zero. The column was then washed with a high-salt buffer (50 mM sodium phosphate, 500 mM NaCl, 0.01% (w/v) Triton X-100, pH 7.2) until the absorbance stabilized. The adsorbed tPA was eluted with a low-pH−high-salt solution (100 mM sodium acetate, 500 mM NaCl, 0.01% (w/v) Triton X-100, pH 2.7). The pH of the eluted tPA was adjusted back to neutrality by adding 1 M sodium phosphate (pH 7.2) to a final concentration of 200 mM.

Purified tPA was dialyzed against 4 L of water in 6000−8000 Da molecular weight cut-off dialysis tubing (Spectrum, Houston, TX) overnight at 4 °C. L-Arginine was added to the dialyzed tPA at a concentration of 0.55 M to keep the tPA soluble at higher concentrations. The samples were then concentrated in Centriprep-10 centrifugal concentrators (Amicon) to a tPA concentration of approximately 1 mg/mL. The final tPA concentration was determined by ELISA.

tPA ELISA. The concentration of tPA was determined using a sandwich ELISA, as described previously (Lin et al., 1993). Briefly, 96-well plates (Falcon Probind #3915; Becton Dickinson, Lincoln Park, NJ) were coated with polyclonal goat anti-tPA IgG (American Diagnostica) and blocked with 1 g/L BSA and 0.5 g/L Tween 20. Supernatant samples or standards and horse radish peroxidase-conjugated goat anti-tPA IgG were added to the wells simultaneously. The horse radish peroxidase-conjugated goat anti-tPA IgG was prepared from polyclonal goat anti-tPA IgG and activated horseradish peroxidase (Pierce, Rockford, IL) according to the manufacturer's instructions. The well-plate was developed with *o*-phenylenediamine, and the absorbance was read at 405 nm on a Lambda plate reader (Perkin Elmer, Wilton, CT). Each tPA concentration reported is the average of two determinations.

SDS-PAGE. Purified tPA was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) under reducing conditions as described previously (Lin et al., 1993). Samples containing 10 μg of tPA were prepared by adding an equal volume of 2× Laemmli buffer (100 mM Tris, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol, pH 6.8) and boiling for 5 min. Samples were loaded onto the gel and electrophoresed for 6 h (1 h at 100 V; 5 h at 300 V) in 1× running buffer (3 g/L Tris base, 14.4 g/L glycine, and 1 g/L sodium dodecyl sulfate). Upon completion, the bands were either visualized by silver staining (Bio-Rad silver stain kit) or blotted onto nitrocellulose.

Western Blots. SDS-PAGE gels were electroblotted onto nitrocellulose (Bio-Rad) using a Hoeffer (San Francisco, CA) TE 62X Transphor II system according to the instructions for the apparatus. The transfer was performed for 3 h at 1.5 A in Dunn carbonate buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol, pH 9.9). The nitrocellulose sheet was allowed to dry overnight. The sheet was then blocked with 5 mg/mL BSA in PBS for 1.5 h at room temperature, and washed three times for 5 min with 0.1% Tween 20 in PBS.

For tPA immunoblots, the nitrocellulose sheet was probed sequentially with goat anti-human uterine tPA IgG (American Diagnostica) and rabbit anti-goat IgG-horseradish peroxidase conjugate (Sigma, No. A5420).

For GNA blots, the nitrocellulose sheet was probed with GNA (*Galanthus nivalis*) lectin−horseradish peroxidase conjugate (EY Laboratories, San Mateo, CA).

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After probing, the blots were developed with the chemiluminescent detection kit (ECL kit; Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Western blots were analyzed by densitometry on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, part number PB95-500195GEI).

Isoelectric Focusing Gels. Isoelectric focusing was performed using a Bio-Rad model 115 isoelectric focusing apparatus. The acrylamide solution was prepared by mixing 2.4 g of urea, 0.25 mL of ampholytes (Bio-Rad), 0.5 mL of 40% acrylamide (29:1 acrylamide to bis ratio; Amresco, Solon, OH), 1.51 mL of water, and 1.0 mL of 10% Triton X-100 (stored at 4 °C with a mixed ion-exchange resin). The mixture was degassed for 5 min, and 25 μL of 0.1% (w/v) riboflavin 5′-phosphate (Bio-Rad), 15 μL of 10% (w/v) ammonium persulfate, and 3 μL of TEMED were added. The gel was cast according to the manufacturer's instructions. TPA samples were diluted to 250 μg/mL in water and 1 μL (0.25 μg) was loaded onto the gel. After 10 min, the gel was placed in the electrophoresis apparatus and electrophoresed for 1.5 h (15 min at 100 V, 15 min at 200 V, and 1 h at 450 V). After electrophoresis, the gel was removed and stained with a silver stain kit (Bio-Rad) according to the manufacturer's instructions.

Monosaccharide Analysis. The monosaccharide content of tPA was determined using the technique of high-pH anion exchange chromatography with pulsed amperometric detection (HPAE-PAD; Hardy et al., 1988), using a Dionex (Sunnyvale, CA) BioLC system with a CarboPac PA1 column and a PAD II detector.

Approximately 100 μL of concentrated tPA was diluted into 3.5 mL of 5 mM trifluoroacetic acid (TFA) or 10 mM HCl and concentrated in Centricon-10 centrifugal concentrators (Amicon) to approximately 1 mg/mL. The final tPA concentration was determined by tPA ELISA. Neutral sugars were released by adjusting the 5 mM TFA samples to 2 M TFA with 4 M TFA; the samples were hydrolyzed at 100 °C for 3 h. Amino sugars were released by adjusting the 10 mM HCl samples to 6 M HCl with 12 M HCl, and hydrolyzing at 100 °C for 4 h. These hydrolysis times were chosen because they were found to release the maximal amount of monosaccharide (Kimura, 1996). Sialic acids were released by adjusting the 10 mM HCl samples to 0.5 M HCl and hydrolyzing at 80 °C for 30 min (Weitzhandler et al., 1993). All samples were cooled and the acid was removed in a Speed-Vac (Savant, Holbrook, NY) with heat.

The lyophilized samples were dissolved in 100 μL of water and injected (75 μL) into the BioLC system. Buffers for chromatography were degassed by sparging with helium for 30 min. Neutral and amino monosaccharides were analyzed at an isocratic NaOH concentration of 16 mM for 25 min. The column was regenerated with 200 mM NaOH for 15 min and then equilibrated with 16 mM NaOH for 20 min. Sialic acids were analyzed using a linear sodium acetate gradient from 5−200 mM over 20 min, with a constant NaOH concentration of 100 mM. The column did not require regeneration after each injection for sialic acid analysis. The pulsed amperometric detector was set to the following conditions: $E_1 = 0.05$ V, $t_1 = 480$ ms; $E_2 = 0.60$ V, $t_2 = 120$ ms; $E_3 = -0.60$ V, $t_3 = 60$ ms.

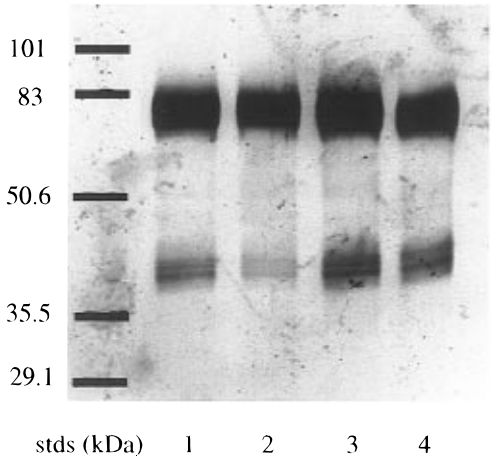

Figure 1. Immunoblot of tPA for the 250 mmHg−310 mOsmol/kg (II) and 250 mmHg−376 mOsmol/kg (I) experiments. Lanes: (1) control (36 mmHg−310 mOsmol/kg; for the 250 mmHg−310 mOsmol/kg (II) experiment), (2) 250 mmHg−310 mOsmol/kg (II), (3) control (36 mmHg−310 mOsmol/kg; for the 250 mmHg−376 mOsmol/kg (I) experiment), and (4) 250 mmHg−376 mOsmol/kg (I).

Results

Purified tPA from cells incubated in serum-free medium for 2 days was analyzed for molecular weight by immunoblots. SDS-PAGE gels were blotted onto nitrocellulose and probed with an anti-tPA antibody. TPA appears as several bands on SDS-PAGE gels. One-chain tPA has a molecular weight of approximately 68 kDa. Because of the two glycoforms (type I and II), one-chain tPA is observed as a double band with a difference in molecular weight (MW) of approximately 2 kDa. Two-chain and degraded tPA appears as several bands in the 35−37 kDa MW range.

Figure 1 shows representative immunoblots of tPA from experiments at 250 mmHg−310 mOsmol/kg (II) and 250 mmHg−376 mOsmol/kg (I). No changes were detectable in the distribution of type I and type II tPA based on the one-chain tPA bands in any of the experiments (Figure 1; replicate immunoblots and densitometry plots in Kimura (1996)). A small amount of two-chain and degraded tPA was detected in samples from the 140 mmHg−310 mOsmol/kg (I), 250 mmHg−310 mOsmol/kg (I and II), and 250 mmHg−376 mOsmol/kg (I) experiments, but this was less than 15% of total tPA (Table 1). Samples from the 250 mmHg−310 mOsmol/kg (III) and especially the 250 mmHg−376 mOsmol/kg (II) experiments (including the respective controls) all contained greater amounts of two-chain and degraded tPA. These cultures were incubated for 2.5 days compared to 2 days for the other experiments, possibly resulting in the death of some cells and the release of intracellular proteases (Gramer and Goochee, 1993). No changes were observed in the distribution of type I and type II tPA for any of these samples (Kimura, 1996).

Western blots of tPA were probed with the lectin GNA, which binds to high-mannose oligosaccharides (Shibuya et al., 1988). GNA blots are shown in Figure 2 for the same experiments as the immunoblots shown in Figure 1. The percentage of two-chain and degraded tPA for all GNA blots is summarized in Table 1. Also included in Table 1 is the ratio of the GNA blot optical density to that of the immunoblot for all cultures; this ratio corrects for differences in the tPA concentration between different samples. For most of the experiments, similar GNA/immunoblot ratios were obtained for the high-pCO_2 and control cultures. However, there are several exceptions to this general finding. The first type of exception is

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Table 1. Summary of Densitometry Results for the Immunoblots and the GNA Blots

condition[a]	immunoblots %2-chain[b]	GNA blots %2-chain[b]	GNA/immunoblot ratio[c] 1-chain	GNA/immunoblot ratio[c] 2-chain[b]
140 mmHg−310 mOsmol/kg (I)	10	0	1.20	0
control for 140−310 (I) (36 mmHg−310 mOsmol/kg)	11	0	1.24	0
250 mmHg−310 mOsmol/kg (I)	13	0	1.16	0
control for 250−310 (I) (36 mmHg−310 mOsmol/kg)	10	0	1.04	0
250 mmHg−310 mOsmol/kg (II)	8	1	0.87	0.09
control for 250−310 (II) (36 mmHg−310 mOsmol/kg)	13	5	1.40	0.49
250 mmHg−310 mOsmol/kg (III)	20	nd[d]	11.3[e]	nd
control for 250−310 (III) (36 mmHg−310 mOsmol/kg)	33	nd	13.6	nd
250 mmHg−376 mOsmol/kg (I)	14	13	0.77	0.74
control for 250−376 (I) (36 mmHg−310 mOsmol/kg)	14	6	0.65	0.22
250 mmHg−376 mOsmol/kg (II)	54	nd	nd	nd
control for 250−376 (II) (36 mmHg−310 mOsmol/kg)	47	nd	nd	nd

[a] Roman numerals indicate independent experiments for each set of conditions. The relevant controls are given below each experiment. [b] All bands in the 40 kDa region of the blots (two-chain and degraded tPA) are included as two-chain. [c] GNA blot optical density divided by the immunoblot optical density. [d] nd, not determined. The splotchy background of this GNA blot (250 mmHg−310 mOsmol/kg (III) and 250 mmHg−376 mOsmol/kg (II)) was such that only the optical densities of the one-chain bands for the 250 mmHg−310 mOsmol/kg (III) experiment could be determined quantitatively. However, visual comparison of the GNA blot and the immunoblot for these experiments did not reveal any noticeable differences in the relative amounts of one-chain tPA for the 250 mmHg−376 mOsmol/kg (II) experiment or of two-chain tPA for either experiment. [e] The dark background of this GNA blot (250 mmHg−310 mOsmol/kg (III) and 250 mmHg−376 mOsmol/kg (II)) was such that a transparency adapter had to be used to scan the gel. Thus, the optical densities of the one-chain bands for the 250 mmHg−310 mOsmol/kg (III) experiment are much greater than those of the immunoblot, which was scanned without a transparency adapter.

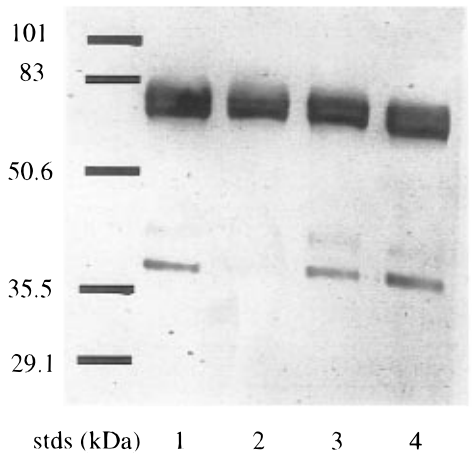

Figure 2. GNA blot of tPA for the 250 mmHg−310 mOsmol/kg (II) and 250 mmHg−376 mOsmol/kg (I) experiments shown in Figure 1. Lane designations are the same as in Figure 1.

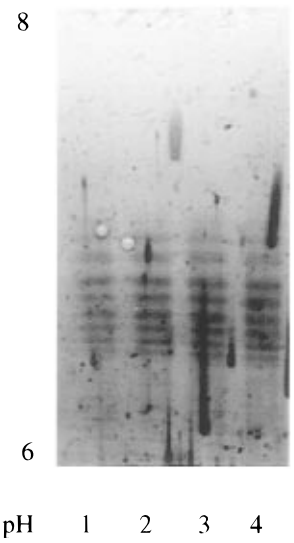

Figure 3. Isoelectric focusing gel of tPA for the 250 mmHg−310 mOsmol/kg (II) and 250 mmHg−376 mOsmol/kg (I) experiments shown in Figures 1 and 2. Lane designations are the same as in Figure 1.

different GNA/immunoblot ratios for two-chain and degraded tPA for high-pCO_2 vs control cultures in the 250 mmHg−310 mOsmol/kg (II) and 250 mmHg−376 mOsmol/kg (I) experiments. We attribute this in part to a lower sensitivity of GNA staining for the two-chain bands when they are present at low levels. This is clearly evident for the 140 mmHg−310 mOsmol/kg (I) and 250 mmHg−310 mOsmol/kg (I) experiments, for which no GNA staining was observed for the two-chain bands, even though the immunoblots showed ≥10% two-chain tPA (Table 1). It is also evident for the 250 mmHg−310 mOsmol/kg (II) experiment shown in Figures 1 and 2. The second type of exception is evidenced in the 250 mmHg−310 mOsmol/kg (II) experiment. In this case, the GNA/immunoblot ratio for one-chain tPA in the high-pCO_2 culture was only 62% of that in the control culture. This suggests a decrease in the content of high-mannose oligosaccharides at high pCO_2. However, the other two 250 mmHg−310 mOsmol/kg cultures had GNA/immunoblot ratios for one-chain tPA that were 112% and 83% of the control, respectively.

The charge heterogeneity of tPA was examined using isoelectric focusing gels. Figure 3 shows IEF gels, analyzed on a pH 6−8 gradient, for the experiments shown in Figures 1 and 2. No significant changes were evident in the distribution of bands from tPA produced at high pCO_2 either visually (Figure 3) or by densitometry (Kimura, 1996). Similar results were obtained for replicate experiments at 250 mmHg−310 mOsmol/kg and 250 mmHg−376 mOsmol/kg (Kimura, 1996).

Data from the monosaccharide analysis experiments are summarized in Table 2. There was some experiment-to-experiment variation in the content of neutral and amino monosaccharides, especially for GlcNAc, in the three control culture analyses. However, comparison of the monosaccharide content ratios for parallel elevated pCO_2 and control samples reveals no significant changes due to elevated pCO_2 in the neutral or amino monosaccharide content of tPA.

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Table 2. Monosaccharide Analysis Data

condition[a]	monosaccharide content (mol/mol of tPA)[b]					
	Fuc	GlcNAc	Gal	Man	Sia	%Neu5Gc
250 mmHg−310 mOsmol/kg (II)	0.5 ± 0.2	4.8 ± 0.5	1.1 ± 0.2	3.1 ± 0.6	nd[c]	nd
control for 250−310 (II) (36 mmHg−310 mOsmol/kg)	0.7 ± 0.1	5.4 ± 0.7	1.6 ± 0.2	3.6 ± 0.6	nd	nd
ratio of 250−310 (II)/control[d]	0.7	0.9	0.7	0.9	nd	nd
250 mmHg−310 mOsmol/kg (III)	0.5 ± 0.0	8.5 ± 1.0	1.7 ± 0.0	3.3 ± 0.1	4.6 ± 0.4	2.2 ± 0.3
control for 250−310 (III) (36 mmHg−310 mOsmol/kg)	0.5 ± 0.1	8.6 ± 0.1	2.0 ± 0.7	4.2 ± 1.7	5.0 ± 0.0	4.0 ± 0.4
ratio of 250−310 (III)/control	1.0	1.0	0.9	0.8	0.9	0.6
250 mmHg−376 mOsmol/kg (II)	0.2 ± 0.1	7.4 ± 0.4	1.9 ± 0.2	4.3 ± 0.5	4.1 ± 0.1	2.0 ± 0.3
control for 250−376 (II) (36 mmHg−310 mOsmol/kg)	0.3 ± 0.2	8.1 ± 0.3	2.0 ± 0.4	4.6 ± 1.3	4.7 ± 0.1	3.2 ± 0.6
ratio of 250−376 (II)/control	0.7	0.9	1.0	0.9	0.9	0.6
250 mmHg−376 mOsmol/kg (III)	nd	nd	nd	nd	2.6 ± 0.0	1.5 ± 0.0
control for 250−376 (III) (36 mmHg−310 mOsmol/kg)	nd	nd	nd	nd	2.3 ± 0.1	2.3 ± 0.1
ratio of 250−376 (III)/control	nd	nd	nd	nd	1.1	0.7
Vehar et al. (1986)	2.5	7.4	3.7	9.8	2.7	nd

[a] The designations for each condition are as in Table 1. [b] Fuc, fucose; GlcNAc, *N*-acetylglucosamine; Gal, galactose; Man, mannose; Sia, total sialic acid (both Neu5Ac and Neu5Gc); %Neu5Gc, Neu5Gc as a percentage of total sialic acid. [c] Not determined. [d] Mean value for the high-pCO_2 conditions divided by the mean value for the respective control.

Results for the total amount of sialic acid per mole of tPA and the amount of *N*-glycolylneuraminic acid as a percentage of total sialic acids (the balance is *N*-acetylneuraminic acid) are also summarized in Table 2. Again, there was variation in the total sialic acid content for the different control samples, but no consistent trend was observed for the total amount of sialic acid on tPA at elevated pCO_2 vs that for the respective controls. However, the percentage of *N*-glycolylneuraminic acid was consistently lower at 250 mmHg pCO_2 (~60% of the control culture value). Although these results are not statistically significant due to the small number of samples, the same pattern was observed in all three experiments.

Discussion

The data presented here indicate that no changes occur in the distribution of type I and type II tPAs (immunoblots), the expression of high-mannose oligosaccharides (GNA blots), or the distribution of surface charges (IEF gels) due to elevated pCO_2.

Earlier, we postulated that some effects of elevated pCO_2 may be due to alterations in intracellular pH. Ammonia can inhibit the sialylation of proteins (Thorens and Vassalli, 1986; Anderson and Goochee, 1995), most likely due to a change in the *trans* Golgi pH that alters the activity of sialyltransferases. Because of this, we believed that elevated pCO_2 might also affect sialylation by altering the *trans* Golgi pH. However, we saw no effect of elevated pCO_2 on total sialic acid content. Anderson and Goochee (1995) found that the sensitivity of O-linked sialylation to ammonia in CHO cells varied with the linkage of the sialic acid. The O-linked α2,6 sialyltransferase was inhibited by ammonia, but the O-linked α2,3 sialyltransferase was not. Clearly, either elevated pCO_2 does not affect *trans* Golgi pH significantly or the N-linked α2,3 sialyltransferase (CHO cells do not express N-linked α2,6 sialic acids) is not sensitive to pH changes.

Besides sialic acid content, changes in the surface charge of tPA could arise from differential removal of a propeptide sequence that contains a positively charged amino acid (Berg and Grinnell, 1991) or deamidation of asparagine residues (Liu, 1992). TPA is secreted with the three-peptide leader sequence Gly-Ala-Arg (GAR), which can be removed by extracellular proteolysis. Deamidation occurs primarily at asparagine residues with a C-flanking glycine or serine (Liu, 1992). The neutral asparagine residue is converted to a negatively-charged aspartate or isoaspartate residue. Heterogeneity in the charge distribution of tPA is evidenced by the presence of at least eight bands in the IEF gels (Figure 3). Since there were no significant changes in the intensities of the bands at elevated pCO_2, our results suggest that there was little change in the charge distribution.

In comparison to published data on the monosaccharide content of tPA from recombinant CHO cells (Vehar et al, 1986), the values obtained here are about 50% lower for each of the three neutral monosaccharides (galactose, mannose, and especially fucose; Table 2). In contrast, the values for *N*-acetylglucosamine and total sialic acids are similar to or greater than those of Vehar et al. (1986). Since all three neutral monosaccharides were obtained from the same tPA sample and TFA hydrolysis step, it appears that the recovery of neutral monosaccharides from the TFA hydrolysate was suboptimal. This is particularly clear when comparing the galactose and sialic acid contents shown in Table 2. Sialic acids in N-linked oligosaccharides are attached to galactose residues, but we detected at least twice as many sialic acid residues as galactose residues. The larger relative discrepancy for fucose may be due in part to a loss of the extremely acid-labile O-linked fucose during tPA purification (Harris et al., 1991). The fact that no differences were seen in the amounts of neutral monosaccharides in parallel high pCO_2 and control cultures suggests that little change occurred in the tPA monosaccharide content. However, it should be noted that differences in monosaccharide content could have been masked by the low recovery of the neutral sugars or by differences in monosaccharide recovery from differently glycosylated tPA molecules.

The only consistent change observed under elevated pCO_2 conditions was in the type of sialic acid on tPA, specifically a decrease in the amount of *N*-glycolylneuraminic acid (Neu5Gc) as a percentage of total sialic acids. Sialic acids are a class of monosaccharide consisting of *N*-acetylneuraminic acid and its derivatives. *N*-Acetylneuraminic acid is hydroxylated on the 5-carbon to form *N*-glycolylneuraminic acid. Our results of lower Neu5Gc content at elevated pCO_2 are similar to those of Grampp et al. (1994), although the initial percentage of Neu5Gc in our experiments was lower (2−4% vs 10−13%). Our initial percentage of Neu5Gc was closer to the 3−5% found by Hokke et al. (1990) on several CHO-derived recombinant proteins.

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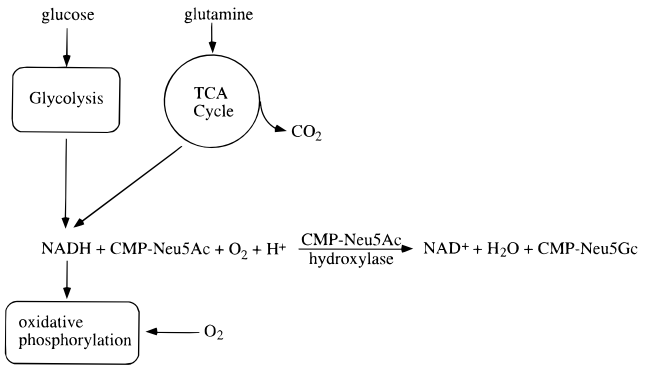

Figure 4. Biosynthesis of *N*-glycolylneuraminic acid (adapted from Shaw and Schauer (1989) and Stryer (1988).

The observed changes in Neu5Gc content are unlikely to have any clinical implications. Human cells in general do not express Neu5Gc (Yuppy and Gottschalk, 1972). Humans who have tumors that express Neu5Gc develop antibodies against Neu5Gc (Higashi et al., 1985). The question as to whether the levels of Neu5Gc reported in CHO-derived glycoproteins can induce antibody expression has not been conclusively resolved. Noguchi et al. (1995) attempted to examine this issue using chickens as a model system. Chickens, like humans, do not express Neu5Gc, and exposure to Neu5Gc can induce anti-Neu5Gc antibodies. Injection of erythropoietin containing approximately 1% Neu5Gc did not induce an immune response in chickens. We are not aware of any reports of antibody production against Neu5Gc due to treatment with CHO-derived glycoproteins. Since exposure to elevated pCO_2 further decreases the amount of Neu5Gc, it would not be expected to lead to an increase in immunogenicity.

The altered expression of Neu5Gc does, however, reveal some clues as to how cells respond to elevated pCO_2. The donor of *N*-glycolylneuraminic acid, CMP-Neu5Gc, is synthesized in the cytosol from CMP-Neu5Ac by the enzyme CMP-Neu5Ac hydroxylase (Shaw and Schauer, 1988, 1989; Muchmore et al., 1989). CO_2 may inhibit the synthesis of *N*-glycolylneuraminic acid by one of two mechanisms (Figure 4). First, the activity of CMP-Neu5Ac hydroxylase may be decreased by alterations in the cytosolic pH. Second, under elevated pCO_2, the supply of NADH may become limited. NADH limitation could be due to a decrease in glucose and/or glutamine consumption (Grampp et al., 1994) and/or to an increase in oxygen consumption. Analysis of the intracellular pH; glucose, glutamine, and oxygen consumption rates; and intracellular NADH concentration would be necessary to draw any definitive conclusions regarding the correct mechanism(s). Results from hybridoma cells exposed to 250 mmHg pCO_2 in well-plate experiments are consistent with a role for decreased glucose consumption (Kimura, 1996).

The data presented here indicate that few if any changes occurred in glycosylation site occupancy (type I vs type II), expression of high-mannose oligosaccharides, or the distribution of surface charges on tPA in response to elevated pCO_2 with or without osmolality control. This was true even though cell growth (30−40% inhibition at 250 mmHg) and tPA production (40% inhibition at 250 mmHg−310 mOsmol/kg) have been shown to be affected (Kimura and Miller, 1996) and suggests robust glycosylation of tPA by CHO cells. We are not aware of any reports of altered tPA glycosylation in response to any other environmental stress.

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