Studies on the turnover of glucocerebrosidase in cultured rat peritoneal macrophages and normal human fibroblasts

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The kinetics of glucocerebrosidase synthesis and degradation in rat peritoneal macrophages and in human fibroblasts have been studied using conduritol B epoxide (CBE), an irreversible and specific inhibitor of mammalian glucocerebrosidase. In cultured fibroblasts, higher concentrations of CBE and/or longer times were required for inhibition of glucocerebrosidase than were necessary for inhibition of the macrophage enzyme. However, inhibition of activity in cell extracts from both cell types showed identical time and concentration dependence. After the removal of CBE from cultures, enzyme activity returned to normal with a half-time of 48 h for macrophages and 40 h for fibroblasts. The reappearance of enzyme activity was prevented by an inhibitor of protein synthesis. Both the rate of synthesis and degradation of glucocerebrosidase enzyme protein were independent of the presence of CBE. The calculated rate of degradation of glucocerebrosidase was confirmed using metabolically labelled enzyme in cell cultures. The rate of synthesis for macrophages is 1.8 ng enzyme h^{-1} mg cell protein^{-1} and the rate of degradation is 1.4% h^{-1} (0.014 h^{-1}). These values were 2.0 ng h^{-1} mg^{-1} and 0.018 h^{-1} for fibroblasts.

Glucocerebrosidase (glucosylceramide glucohydrolase) is a membrane-bound lysosomal hydrolase, which catalyzes the hydrolysis of glucocerebroside, a constituent of biological membranes. The enzyme has been purified to homogeneity [1, 2], extensively characterized [3–9] and its gene isolated [10–15]. The activity of the enzyme is markedly decreased in the tissues of patients with Gaucher disease, a recessive inherited disorder characterized by the accumulation of glucocerebroside in the cells of the reticuloendothelial system [16]. Several mutations in the gene for glucocerebrosidase result in different clinical subtypes of the disease [17, 18]. Correlations between phenotypes of Gaucher disease, clinical severity and residual enzyme activity in tissues and cells have so far been inadequate [19] (for reviews see [20, 21]). However, there are some suggestions that there is less activity and less enzyme cross-reactive material in some of the phenotypes of Gaucher disease [22, 23]. The rate of synthesis or degradation of the enzyme may be altered for the mutant proteins and, as such, may be a clue to understanding the widely different phenotypes of Gaucher disease. The present report describes the kinetics of glucocerebrosidase synthesis and degradation in cultured human fibroblasts from normal subjects and rat peritoneal macrophages using conduritol B epoxide, a specific and irreversible inhibitor of glucocerebrosidase [24, 25].

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Abbreviations. CBE, conduritol B epoxide; SDS, sodium dodecyl sulfate.

Enzyme. Glucosylceramide glucohydrolase (EC 3.2.1.45).

MATERIALS AND METHODS

Materials

Conduritol B epoxide (CBE) was prepared according to Legler [26]. D-[1-^{14}C]Glucosylceramide (0.34 Ci/mol) was synthesized according to Brady et al. [27]. The following compounds and materials were purchased from the indicated sources: MEM α medium from Grand Island Biological Co. (N.Y.); McCoy's 5A and Dulbecco's minimum essential medium from M. A. Bioproducts (Walkersville, MD); [^{35}S]-methionine (1185 Ci/mmol) from ICN Pharmaceuticals Inc.; thioglycollate from BBL (Cockeysville, MD); iodoacetamide from Aldrich Chemical Co.; triethanolamine from Fisher Scientific Co. and Protein-A–Sepharose from Pharmacia Fine Chemicals (Uppsala, Sweden).

Cell cultures

Media composition. α-Mops: MEM α medium (Gibco 430-1900) was supplemented with 0.4 g/l NaCl, 4.01 g/l 3-*N*-[morpholino]propanesulfonic acid (Mops), and 2.25 ml/l NaOH (5 M), final pH 7.2. α-10: MEM α medium was supplemented with 2.2 g/l $NaHCO_3$, 10% fetal calf serum, pH 7.2. Both media contained 100 U penicillin and 100 µg streptomycin/ml.

Macrophages were collected by peritoneal lavage from rats (Sprague-Dawley, 150–200 g) given intraperitoneal injections of 10 ml 10% thioglycollate broth 5 days before harvest as described earlier [28]. After killing by CO_2 inhalation, exudate cells were harvested from the peritoneal cavity

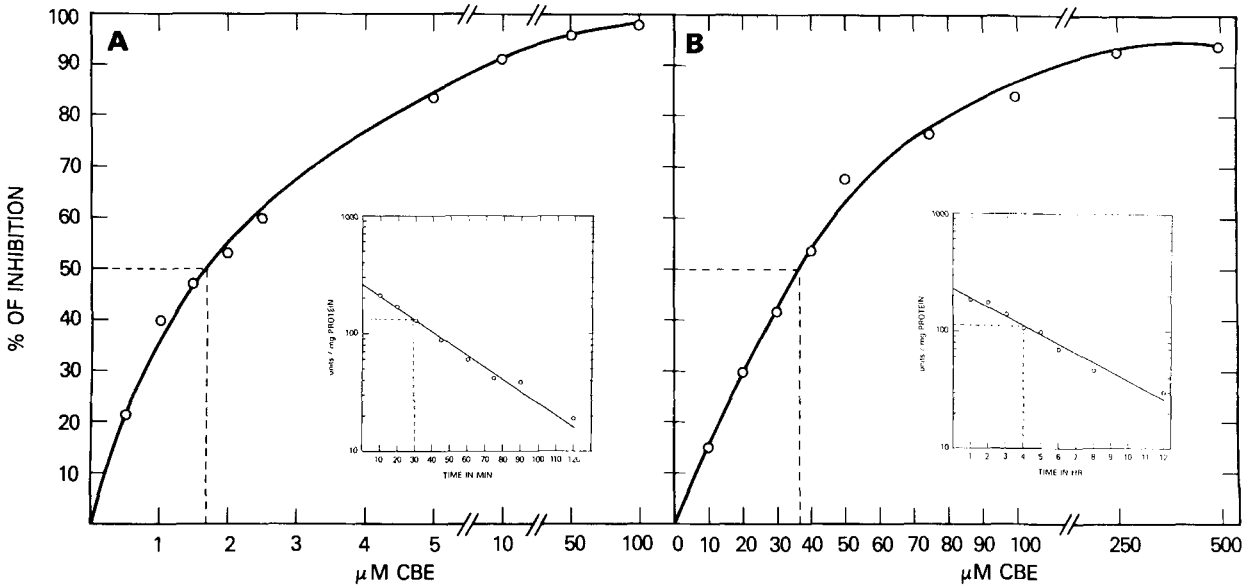

Fig. 1. In vivo *inhibition of macrophage and fibroblast glucocerebrosidase by CBE.* Macrophage and fibroblast monolayers were incubated at 37°C for 2 h with various concentrations of CBE. Cells were then harvested and washed with NaCl/P_i, extracts were made and glucocerebrosidase was assayed in the cell extract. Enzyme activity was normalized to cell protein and inhibition was calculated as percentage of control untreated cells. (A) Macrophages, 1.75 μM CBE for 50% inhibition. (B) Fibroblasts, 36 μM for 50% inhibition. Inserts: kinetics of inhibition at 10 μM CBE. Cells were preincubated with 10 μM CBE for indicated period and then assayed for activity as described above. (A) Macrophages, $t_{1/2}$ of inactivation = 30 min. (B) Fibroblasts, $t_{1/2}$ of inactivation = 4 h

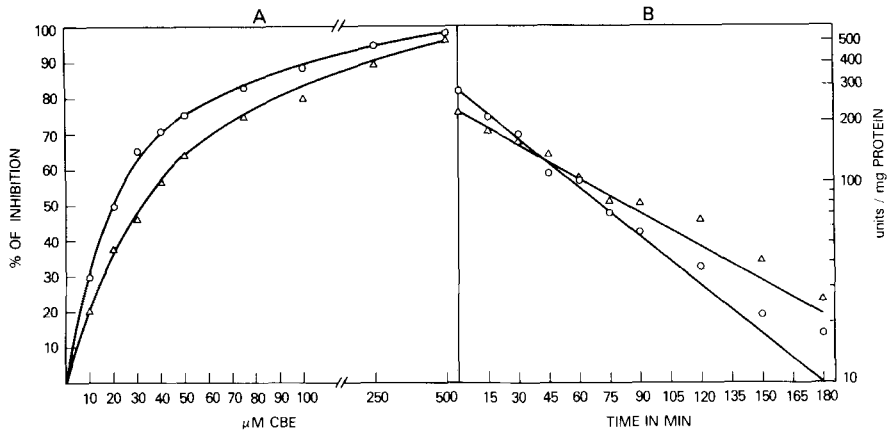

Fig. 2. In vitro *inhibition of macrophage (○——○) and fibroblast (△——△) glucocerebrosidase by CBE.* Cell extracts were assayed for glucocerebrosidase in the presence of indicated concentration of CBE. (A) Dose effect of CBE without preincubation. (B) Kinetics of inhibition by CBE. Cell extracts were preincubated with 50 μM CBE for indicated period and then assayed for activity

in α-Mops medium and plated into tissue-culture dishes with a 35-mm-diameter well (no. 3506 Costar, Cambridge, MA). After incubation for 2 h at 37°C, non-adherent cells were removed by thorough washing. The remaining adherent macrophages were cultivated for 24 h at 37°C in α-10 medium in an incubator (5% CO_2/air) before further studies. Cultures prepared in this manner contained more than 95% macrophages, as judged by their ability to take up latex beads and by morphology.

Normal human foreskin fibroblasts were obtained from Meloy Laboratories (Springfield, VA). Cells were grown in McCoy's 5A medium containing 10% fetal calf serum, gentamycin (100 μg/ml) and fungizone (2.5 μg/ml), at 37°C in an atmosphere of 95% air/5% CO_2.

Inhibition studies with CBE

CBE, freshly prepared as a stock solution of 1 mM in either α medium containing 10% fetal calf serum or McCoy's medium containing 10% fetal calf serum was added to macrophage or fibroblast cell cultures at the concentrations indicated. After incubating the cells for various time periods the medium was aspirated and monolayers were rinsed three times with phosphate-buffered saline (NaCl/P_i). Cells were then harvested with a rubber policeman and washed with NaCl/P_i. Cell extracts were made by suspending the pellet in buffer (0.1 M citrate/phosphate buffer, pH 6.0 containing 0.2% Triton X-100), sonicating briefly (10 s) in a cell disrupter (Heat Systems, Ultrasonics Inc.) and then centrifuging at 48000 × *g* for 20 min. Glucocerebrosidase was assayed in the supernatant with [1-^{14}C]glucosylceramide according to the method described [2].

Immunoprecipitation studies

Cellular glucocerebrosidase was immunoprecipitated with polyclonal rabbit anti-glucocerebrosidase antibodies raised

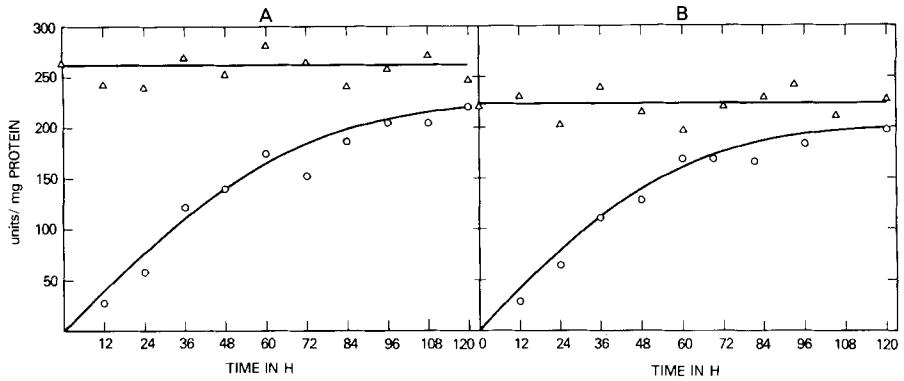

Fig. 3. *Time course of reappearance of glucocerebrosidase in CBE-treated cells after removal of CBE.* Macrophage and fibroblast monolayers were treated with 100 μM CBE for 16 h, washed three times with medium and cultured further without CBE. Cells were harvested at different time intervals, washed with $NaCl/P_i$ and extracts were made and assayed for glucocerebrosidase activity. Enzyme activities were normalized to cell protein. (○——○) Cells treated with CBE, (△——△) control cells without CBE treatment. (A) Macrophages, (B) fibroblasts

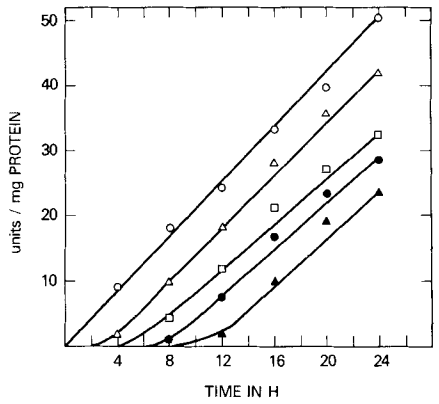

Fig. 4. *Treatment of cells with cycloheximide after removal of CBE.* Macrophage monolayers were first treated with 100 μM CBE for 16 h. Cells were then washed free of CBE, treated with cycloheximide (0.2 mM) for various time periods (2–8 h), washed free of cycloheximide and cultured further up to 24 h. Glucocerebrosidase was assayed in the cell extracts made from cells harvested at various time points after removal of cycloheximide. Cells were treated with cycloheximide for (○——○) 0 h, (△——△) 2 h, (□——□) 4 h, (●——●) 6 h and (▲——▲) 8 h

against purified homogeneous human placental enzyme [2]. Since the antibody does not cross-react with rat glucocerebrosidase (unpublished data), all the immunoprecipitations and studies of the rate of synthesis were done in human fibroblasts. Cells, first starved for 2 h in methionine-free Dulbecco's minimal essential medium containing 10% dialysed fetal calf serum were pulsed for 1 h with [^{35}S]-methionine (262 μCi/ml) in the same medium. Cells were washed twice with $NaCl/P_i$ and the medium was replaced with medium containing methionine. After various lengths of time, cells were harvested, washed with $NaCl/P_i$ and suspended in Tris/HCl, pH 7.0. Cells from one T-25 flask were suspended in 200 μl Tris/HCl (50 mM, pH 7.0) containing 20 mM sodium phosphate and 2.5 mM dithiothreitol and kept frozen. After thawing, 5.25 μl sodium dodecyl sulfate (SDS) (20%) and aprotinin (100 U) were added and the suspension was boiled for 2 min, cooled and sonicated for 30 s. After centrifugation at $50000 \times g$ for 20 min, the supernatant was carefully removed using a thinly drawn-out pasteur pipette. To the supernatant, 65 μl diluting buffer (100 mM Tris, pH 7.4 containing 20% Triton X-100, 300 mM NaCl, 10 mM EDTA and 400 U/ml aprotinin), 6.5 μl iodoacetamide (0.5 M) and 5 μl rabbit anti-glucocerebrosidase serum were added and incubated for 1 h at 37°C, then overnight at 4°C. The suspension was then centrifuged at $50000 \times g$ for 10 min and the supernatant was again carefully removed. 25 μl protein-A–Sepharose beads were added as a 1:1 slurry in $NaCl/P_i$ and the resulting suspensions was incubated at room temperature for 3 h. After centrifugation at $12000 \times g$ for 2 min, supernatant was carefully removed and the beads were washed four times with washing buffer (150 mM triethanolamine, pH 7.4, containing 150 mM NaCl, 0.1% SDS and 5 mM EDTA). Beads were solubilized with 1% SDS in $NaCl/P_i$ and counted.

RESULTS

Inhibition of macrophage and fibroblast glucocerebrosidase by CBE

Inhibition of glucocerebrosidase by CBE in cultured macrophages or fibroblasts was shown to be both time and concentration dependent. However, the rate and extent of inhibition were different for these two cell types. In a 2-h incubation the concentration required for 50% inhibition of the macrophagge enzyme activity was 1.75 μM CBE compared with 36 μM for fibroblasts (Fig. 1). At 10 μM CBE, the $t_{1/2}$ (the time required to inhibit 50% of initial activity) was about 30 min for macrophages and 4 h for fibroblasts (inserts, Fig. 1). Complete inhibition was achieved when both cells were treated with 100 μM CBE for 16 h. When cell extracts from both cells were treated with CBE (Fig. 2), the rate and extent of inhibition were found to be similar (± 10%) suggesting a differential *in vivo* effect of CBE on macrophages and fibroblasts. This may be due to poorer permeability or transport of CBE in fibroblasts compared to macrophages.

Reappearance of glucocerebrosidase activity after CBE treatment

The *in vivo* inhibitory effect of CBE on macrophage and fibroblast glucocerebrosidase was found to be reversible. After completely inhibiting glucocerebrosidase activity by treatment with 100 μM CBE for 16 h and then washing cells free of CBE, the enzyme activity returns slowly as shown in Fig. 3 and reached normal levels approximately 5 days after removal of CBE. Enzyme activity was half normal at 48 h for macrophages and 40 h for fibroblasts.

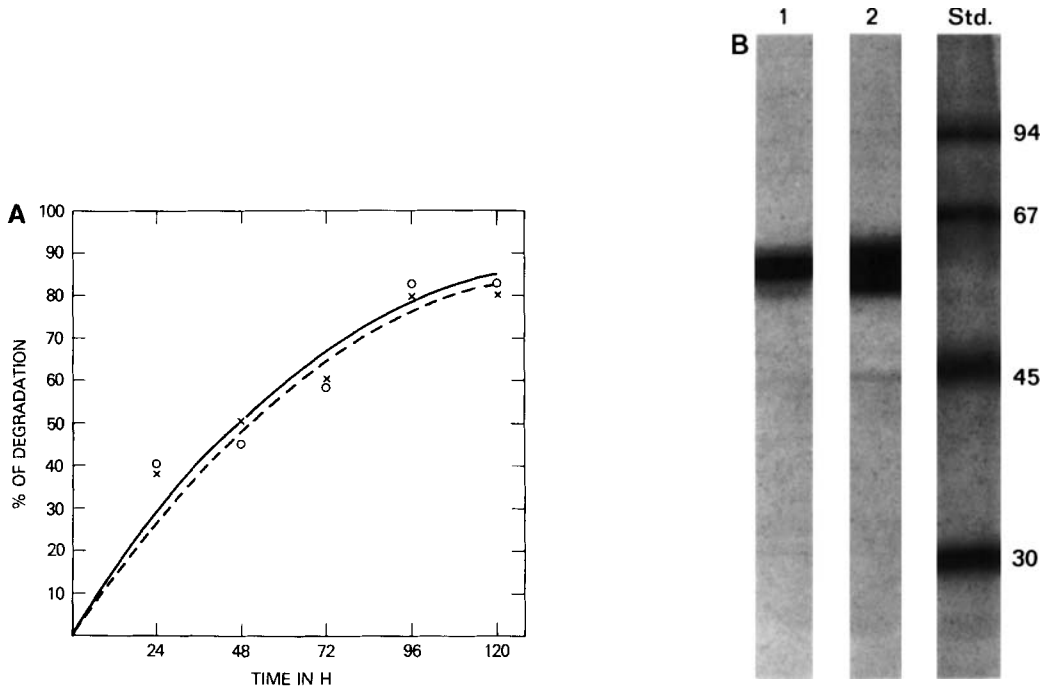

Fig. 5. *(A) Degradation of metabolically labelled glucocerebrosidase in fibroblasts. (B) SDS/polyacrylamide gel electrophoresis of immunoprecipitated glucocerebrosidase.* (A) Cells were pulsed with [^{35}S]methionine (262 μCi/ml) for 1 h, chased and at indicated times the radioactivity incorporated into glucocerebrosidase was determined by immunoprecipitation as described in Materials and Methods. Degradation was calculated as a percentage of glucocerebrosidase radioactivity at 0 time after [^{35}S]methionine pulse. (○——○) No CBE. (×——×) In presence of 10 μM CBE. (B) [^{35}S]Methionine-labelled samples immunoprecipitated as above were electrophoresed on 10% Laemmli gels and autoradiographed as described in [9]. Lane 1, 0 chase; lane 2, 48-h chase. [^{14}C]Carboxymethylated protein standards are included for reference

Table 1. *Relative rate of fibroblast glucocerebrosidase synthesis at intervals after treatment with CBE*
Cells were preincubated with 100 μM CBE for 16 h and then washed free of CBE. At intervals following this washout, cells were pulsed with [^{35}S]methionine (262 μCi/ml) and the radioactivity incorporated into fibroblast glucocerebrosidase and total trichloroacetic-acid-precipitable protein was determined as described in Materials and Methods. The data represent the mean ± SD for triplicate determinations

Time after CBE removal	Fibroblast glucocerebrosidase activity (mean ± SD)	(*a*) Glucocerebrosidase radioactivity (mean)	(*b*) Protein radioactivity (mean)	*a*/*b* X10^3 (mean ± SD)
h	units/mg cell protein	cpm $\times 10^{-3}$/mg cell protein		
No treatment	226 ± 17	8.1	8965	0.90 ± 0.03
0	3 ± 1	9.2	9074	1.01 ± 0.11
17	47 ± 6	8.7	8525	1.02 ± 0.10
24	64 ± 7	8.8	8244	1.07 ± 0.06
46	112 ± 9	8.5	8719	0.97 ± 0.06
70	163 ± 13	8.6	8427	1.01 ± 0.18
94	191 ± 16	8.3	8372	0.99 ± 0.15

The reappearance of glucocerebrosidase activity was shown to represent new synthesis of the enzyme in studies with cycloheximide, a reversible inhibitor of protein synthesis. Cells treated with 100 μM CBE for 16 h were washed and then pulsed with cycloheximide (0.2 mM) for various times (Fig. 4). Reappearance of activity was delayed in proportion to the duration of cycloheximide treatment indicating that new protein synthesis is required for enzyme recovery.

Rates of synthesis and degradation of glucocerebrosidase

Since CBE is an irreversible inhibitor of glucocerebrosidase, the reappearance of activity to a steady-state level can be utilized to calculate the rate of synthesis of the protein. This presumes, however, that the rates of synthesis and degradation are not altered by CBE treatment. This assumption was checked by comparing, in control and CBE-treated cells the amount of [^{35}S]methionine incorporated into glucocerebrosidase at various times after CBE preincubation. No significant differences were observed in the ratios of glucocerebrosidase radioactivity to total protein radioactivity between control and treated cells (Table 1).

Similarly the rate of disappearance of the label in glucocerebrosidase from control and CBE-treated cells was not different (Fig. 5). SDS/polyacrylamide gel electrophoresis followed by autoradiography shows that all radioactivity immunoprecipitated by this method is associated with protein

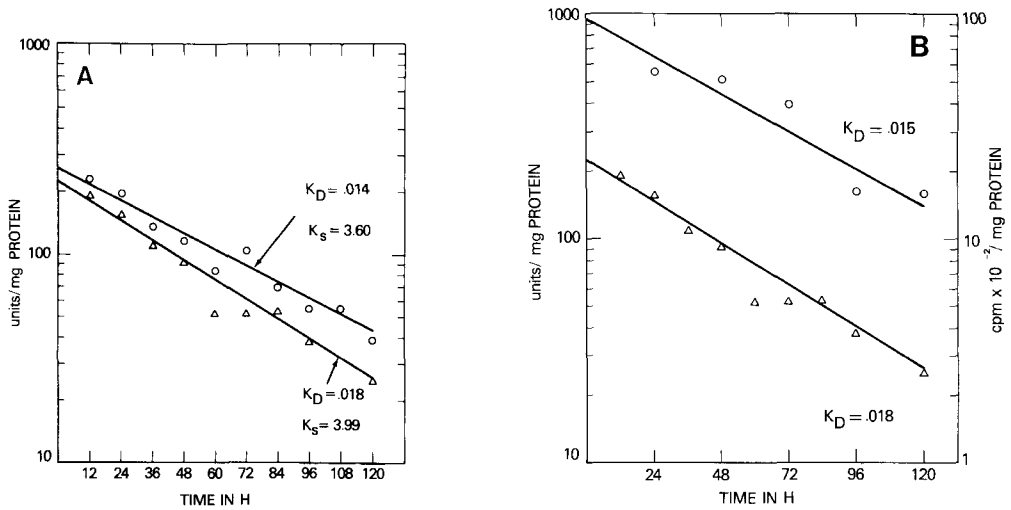

Fig. 6. *(A) Kinetics of macrophage (○——○) and fibroblast (△——△) glucocerebrosidase degradation* in vivo. *(B) Comparison of the kinetics of degradation of fibroblast glucocerebrosidase calculated from the reappearance of enzyme activity after CBE treatment with the kinetics of disappearance of pulse-labeled enzyme radioactivity.* (A) Data from Fig. 3, replotted as log ($c_N - c_t$) versus time, in which c_N is the normal level of glucocerebrosidase in respective cells and c_t is the enzyme level at various times after removal of CBE. Each point represents the average of three experiments. k_D is the first-order constant for glucocerebrosidase degradation, as determined by the slope of the line. k_S, the rate of glucocerebrosidase synthesis, equals $k_D c_N$. (B) (△——△) Semilogarithmic plot of $c_N - c_t$ versus time as described in (A). (○— —○) Semilogarithmic plot of glucocerebrosidase radioactivity immunoprecipitated after [^{35}S]methionine pulse versus time

bands corresponding in molecular mass to glucocerebrosidase [2, 17].

Kinetics of return of glucocerebrosidase activity

The method by which the rate of synthesis and degradation of glucocerebrosidase was determined is that of Price et al. [29], which is based on certain assumptions. The assumptions are (a) that glucocerebrosidase is being synthesized at a constant rate, k_S and (b) that a constant fraction, (k_D), of the active glucocerebrosidase molecules is being degraded per unit of time. From these assumptions the following equation can be derived

$$\frac{dc}{dt} = k_S - k_D c. \quad (1)$$

If $c = 0$ at $t = 0$, a condition obtained before removal of CBE, then

$$c_t = \frac{k_S}{k_D}(1 - e^{-k_D t}) \quad (2)$$

where c_t represents the enzyme level at any time, t, after removal of the inhibitor (CBE).

At the steady-state level, c_N, the rate of glucocerebrosidase synthesis equals the rate of degradation. Hence, if k_D is known, k_S can readily be calculated from the equation:

$$k_S = k_D c_N. \quad (3)$$

A linear transformation of Eqns (2) and (3) permits graphic evaluation of k_D

$$\log_{10}(c_N - c_t) = (-k_D \log_{10} e)t + \log_{10} c_N. \quad (4)$$

In the experiment depicted in Fig. 4 the time course of return of glucocerebrosidase to the steady state after CBE treatment was compared between macrophages and fibroblasts. The difference between the activity at any time after CBE removal and the steady-state value (mean), obtained from control untreated cells, was plotted on a semilogarithmic scale against time (Fig. 6A). k_D was estimated from the slope of the line according to Eqn (4) and k_S from Eqn (3). We have calculated that for macrophages 3.6 units or 1.8 ng glucocerebrosidase mg cell protein^{-1} h^{-1} were being synthesized and 1.4% of the enzyme molecules present were being destroyed in that time ($k_D = 0.014\ h^{-1}$). For fibroblasts these values are 4.0 units or 2.0 ng and 1.8% (0.018 h^{-1}) for synthesis and degradation respectively. For this calculation the specific activity of glucocerebrosidase is assumed to be 2×10^6 units/mg (equivalent to that of the purified placental enzyme [2]). The rate of degradation in fibroblasts ($k_D = 0.015\ h^{-1}$), calculated from the rate of disappearance of ^{35}S label in immunoprecipitated enzyme (Fig. 6B), independently confirms the rate determined from steady-state calculations, thus increasing the confidence in this measurement.

DISCUSSION

CBE, a potent irreversible inhibitor of various plant glucosidases [30], has been shown to be an effective inhibitor of mammalian glucocerebrosidases [24, 25]. We have demonstrated that CBE inhibits glucocerebrosidase in cultured macrophages and fibroblasts to different extents. In contrast, glucocerebrosidase in extracts from both these cell types was found to be equally sensitive to CBE inhibition. This difference most likely is due to poorer permeability or transport of CBE in fibroblasts than in macrophages.

The complete inhibition of macrophage and fibroblast glucocerebrosidase by CBE was found to be followed by a progressive return of the enzyme activity to normal levels in 5 days after removal of CBE from the culture medium. Studies in mice have shown that a single injection of CBE produced near complete inhibition of glucocerebrosidase in various tissues and activity returned to about 80% of normal within 16 days [31]. We have used steady-state assumptions to calculate the rates of synthesis and degradation from the reappearance of enzyme activity after removal of the inhibitor. These calculations assume that the return of activity is due to

new synthesis of enzyme protein rather than reactivation of the enzyme. By use of cycloheximide pulses, the return of enzyme activity was delayed, thus demonstrating the requirement for new protein synthesis in this process. These calculations also rely on the evidence provided earlier [28] that no residual CBE remains after washout to inhibit newly synthesized enzyme and thus prevent accurate measurement of the rate of synthesis of new enzyme molecules. These experiments show that macrophages and fibroblasts have similar rates of synthesis and degradation of glucocerebrosidase implying thereby that the process of turnover of this enzyme is not cell-specific. Both the rates of synthesis and degradation of the enzyme protein were found to be unaffected by the presence of CBE. The ability to independently confirm the rate, determined using the approach to steady-state calculations by specific immunoprecipitation of ^{35}S-labeled enzyme, builds confidence in the ability to use these methods for the measurement of synthesis and degradation in mutant cells. The turnover of lysosomal β-galactosidase was studied earlier in fibroblasts from patients with different types of β-galactosidase deficiency using the suicide substrate β-D-galactopyranosylmethyl-*p*-nitrophenyltriazene [32]. This method described here promises to be of considerable value for studying the turnover rates in the several different mutations of glucocerebrosidase. Similar approaches may contribute to understanding the differences in the mutations of the gene(s) for other enzymes.

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