Treatment of PC12 cells with nerve growth factor increases iron uptake

James MWANJEWE, Betsy King HUI, Michael D. COUGHLIN and Ashok K. GROVER¹

Department of Medicine, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

Phaeochromocytoma PC12 cells treated with nerve growth factor (NGF) differentiate into a neuronal phenotype. Here we compare the uptake of transferrin-bound and non-transferrin-bound iron in NGF-treated (neuronal phenotype) and control (proliferating) PC12 cells. The non-transferrin-bound iron uptake was greater in the NGF-treated cells than in the control, independently of the uptake time, the iron-chelating agents used, the oxidation state of iron (Fe²⁺ or Fe³⁺) and the iron concentration tested. The NGF-treated cells expressed L-type and N-type voltage-operated Ca²⁺ channels. Nitrendipine (an L-type inhibitor) and possibly ω -conotoxin (an N-type inhibitor) inhibited the iron uptake by 20%. Thapsigargin inhibits the endoplasmic reticulum Ca²⁺

pump and allowed Mn²⁺ entry into cells. Preincubating PC12 cells with thapsigargin increased the iron uptake. The rate of transferrin-bound iron uptake was less than 1% of the non-transferrin-bound iron uptake and the maximum transferrin-bound iron uptake was also very low. We conclude that an increase in the iron uptake by multiple pathways accompanies the transition of PC12 cells from the proliferating to the neuronal phenotype.

Key words: ascorbate, dopaminergic neurons, nitric oxide synthase, Parkinson's disease, transferrin.

INTRODUCTION

Iron transport into cells of brain and other tissues occurs via transferrin-bound iron (TBI) and non-transferrin-bound iron (NTBI) pathways and the role of another glycoprotein, p97, has also been described [1–3]. Apotransferrin, an extracellular 80 kDa protein, binds iron very tightly to form holotransferrin, which in turn can bind to transferrin receptors and the complex is taken up by cells by endocytosis [4,5]. The acidic environment of endosomes causes iron to be released; from there it can be transported by another protein termed nramp. NTBI has been suggested to occur via the nramp2 protein (also termed DCT1) located in the plasma membrane in most cells but by nramp1 in macrophages [6–8]. Several other proteins such as SFT1 and HFE have also been proposed to modulate iron transport in mammalian cells. A low-affinity NTBI uptake (K_m 15 μ M) also occurs in erythroid cells, whereas a high-affinity NTBI transport has been reported in many cell types [2,3,9–11]. The high-affinity NTBI transporter carries Fe²⁺ with a K_m of less than 1 μ M, is temperature dependent and is inhibited by La³⁺, Cd²⁺, Co²⁺, Mn²⁺ and Cu²⁺ or by chelation with diethylenetriaminepentaacetate.

Brain iron is an essential cofactor for tyrosine hydroxylase, tryptophan hydroxylase, xanthine oxidase and ribonucleoside reductase [12–14]. Data on the effects of iron deficiency on brain noradrenaline (norepinephrine) suggest that the iron in brain is regulated differently from that in the peripheral tissues [15]. Iron can be transported to the cerebrospinal fluid through the blood–brain barrier and is present in very high concentrations in the substantia nigra, putamen, caudate nucleus, globus pallidus, red nucleus and dentate nucleus regions of the brain [15]. An increase in the iron content of the substantia nigra is associated with severe cases of Parkinson's disease and this association increases with age [12–14]. A decrease in the transferrin receptor sites and in transferrin have been reported in Parkinson's disease. An elevation in brain iron together with a decrease in the

transferrin pathway would suggest that there is an increase in NTBI in Parkinson's disease [15].

The rat adrenergic neural tumour phaeochromocytoma cell line PC12 is used extensively as a model for dopaminergic neurons. PC12 cells treated with nerve growth factor (NGF) exhibit properties of sympathetic neurons: neurite outgrowth and electrical excitability [16]. They express cholinergic receptors, Na⁺-channels, N-type voltage operated Ca²⁺ channels and neuronal nitric oxide synthase (nNOS) [17–19]. They also exhibit high levels of expression of sarcolemmal Ca²⁺ pump and low levels of the organellar pump [20]. Here we characterize the NTBI uptake in PC12 cells and demonstrate that the neuronal phenotype shows greater NTBI uptake than the undifferentiated cells and that the rate of iron uptake through NTBI is much greater than that for TBI uptake.

EXPERIMENTAL

PC12 cultures

PC12 cells in passages 12–30 were cultured on plates coated with bovine achilles-tendon collagen in RPMI 1640 medium supplemented with 5000 i.u. penicillin and 5000 μ g streptomycin, 10% (v/v) fetal calf serum and 5% (v/v) calf serum [20]. At each passage the cells were split 1:4. To treat the cells with NGF, the growth medium containing 40 ng/ml of 2.5 S NGF, purified as previously described, was replenished 2, 5 and 7 days after the initial plating [21]. The cells were harvested in physiological saline solution (PSS) after a further 2 days. PSS contained 10 mM Hepes, 140 mM NaCl, 5 mM KCl, 5 mM MgCl₂ and 1.5 mM CaCl₂, pH 7.4 (pH at 37 °C).

NTBI uptake experiments

Typically NTBI uptake was performed during shaking for 2 min in a 300 μ l reaction mixture at 37 °C in PSS containing specified concentrations of total iron including trace amounts of ⁵⁵FeCl₃ (1–2 μ Ci/ml, specific radioactivity 71 Ci/mmol; Amersham-

Abbreviations used: NGF, nerve growth factor; nNOS, neuronal nitric oxide synthase; NTA, nitrilotriacetate; NTBI, non-transferrin-bound iron; PSS, physiological saline solution; TBI, transferrin-bound iron.

¹ To whom correspondence should be addressed (e-mail groverak@mcmaster.ca).

Pharmacia) and nitrilotriacetate (NTA) or citrate with 0 or 1 mM ascorbate and PC12 cells containing 0.4–0.8 mg of protein. At the specified time, 5 ml of a chilled wash solution [30 mM imidazole/HCl (pH 6.8)/0.5 mM EGTA/250 mM sucrose] was added to the sample and contents of the tube were filtered instantaneously under suction through a 0.45 μ m pore-size nitrocellulose filter presoaked in 1 % BSA and 100 mM KCl. The filter was washed three times with 5 ml of the chilled wash solution. The amount of radioactivity remaining on the filter was determined by scintillation counting. Uptake was performed at 0 and 37 °C; the difference between uptake at the two temperatures was treated as NTBI uptake. Specific variations made in this protocol will be as described in the Results section.

TBI uptake

Human apotransferrin (Sigma Chemical Co.) was labelled with $^{55}\text{FeCl}_3$ and separated from unbound iron by extensive dialysis as previously described [22]. The human and rat transferrins have been shown to give comparable results in rats [22]. The amount of labelled holotransferrin was determined from the level of radioactivity, assuming a 2:1 stoichiometry of iron to transferrin. TBI uptake experiments were performed as for NTBI except that holotransferrin replaced iron and the chelators and ascorbate were not used. Blank values were determined as the radioactivity taken up in the presence of a 1000-fold excess of holotransferrin (Sigma).

Western blots

NGF-treated and control PC12 cells were harvested in 5 ml of chilled Na^+ /Hepes buffer [10 mM Hepes/140 mM NaCl/1.5 mM CaCl_2 (pH 7.4 at 37 °C)], centrifuged at 50 *g* for 10 min and resuspended in 500 μ l of the same buffer but also containing 1 mM PMSF, 1 mM dithiothreitol and 50 μ g/ml leupeptin at 4 °C. The cell suspensions were sonicated and then rapidly centrifuged at 10000 *g* for 1 min. The supernatant was removed and subjected to SDS/PAGE followed by Western blotting as described previously [19]. The nNOS protein was detected with anti-nNOS from Sigma and enhanced chemiluminescence kit from Amersham.

Statistical analysis

Values given throughout are means $\pm$ S.E.M. for *n* replicates. Student's *t* test was used where applicable; and *P* < 0.05 was considered to be statistically significant.

RESULTS

Time course of iron uptake

Figure 1 shows the time course of NTBI uptake in both NGF-treated and control cells at 0 and 37 °C with 0.43 μ M total Fe^{2+} buffered with NTA. There was a small amount of uptake at 0 °C that increased monotonically with time until 10 min. However, the uptake at 37 °C was much greater than at 0 °C. Figure 1 also shows the difference between the uptake at 37 °C and that at 0 °C. As NTBI transport is known to be dependent on temperature, the difference between the values at 37 and 0 °C was taken as the NTBI transport. The NTBI uptake was non-linear with time, indicating that it was saturable. The specific activity of NTBI uptake (nmol uptake/g protein) for the NGF-treated cells was greater than that for the control (*P* < 0.05). Experiments with Fe^{2+} citrate, Fe^{3+} citrate, Fe^{2+} NTA and Fe^{3+} NTA all showed similar time courses. To determine whether the uptake at 2 min represented the initial velocity, we compared the uptakes

Figure 1 Time course of NTBI uptake in NGF-treated and control PC12 cells

NGF-treated (A) or control (B) PC12 cells were incubated in a solution containing 0.43 μ M total iron and NTA at 0 or 37 °C in ascorbate/PSS. Aliquots were filtered after 2, 4, 6, 8 and 10 min. Symbols: Δ , uptake at 37 °C; ∇ , uptake at 0 °C; $\circ$, uptake at 37 °C minus that at 0 °C. All values are means $\pm$ S.E.M. for six replicates. At each time point, the values for the difference between the two temperatures were significantly higher for the NGF-treated cells than for the control cells (*P* < 0.05).

at 0.5 and 2 min. The uptake at 0.5 min was 23 ± 3 % (expected 25 %) of the value for 2 min. The uptake for 2 min was therefore used to monitor the initial velocity of NTBI uptake in the subsequent experiments.

Effects of iron concentration on NTBI uptake

NTA has been used to chelate iron in some studies; citrate has been used in others. To ensure that our results did not depend on the chelating agent used, we conducted experiments with both NTA and citrate. NTBI uptake by NGF-treated PC12 cells was examined at a series of total iron (Fe^{2+} or Fe^{3+}) concentrations by using a 1:4 ratio of iron to NTA or a 1:100 ratio of iron to citrate (Figure 2). These ratios of iron to NTA or citrate have been reported previously to be optimum [2,9–11]. The uptakes at 37 and 0 °C and the difference between the two values, taken as the NTBI uptake, are shown in Figure 2. The linear correlation coefficients of the fits for the Eadie–Hofstee plots for Figures 2(A), 2(B), 2(C) and 2(D) were 0.949, 0.968, 0.931 and 0.917 respectively. Similarly, Hill plots of Figures 2(A), 2(B), 2(C) and 2(D) gave linear correlation coefficients of 0.995, 0.995, 0.993 and 0.987 respectively. The values of Hill coefficients for Figures 2(A) (0.9 ± 0.1), 2(B) (1.2 ± 0.1), 2(C) (1.0 ± 0.1) and 2(D) (1.3 ± 0.2) did not differ significantly from 1 (*P* > 0.05). The maximum uptake was 100 nmol/2 min per g, i.e. 50 nmol/min per g. The K_m values for total iron calculated from these slopes for Figures 2(A), 2(B), 2(C) and 2(D) were 2.6 ± 0.4 , 1.0 ± 0.2 , 1.7 ± 0.3 and 1.6 ± 0.4 μ M respectively. Note that these K_m values correspond to concentrations of total iron and not free iron.

Figure 3 shows a comparison of NTBI uptake in NGF-treated and untreated cells with 0.1, 1 and 10 μ M total iron chelated with NTA or citrate as specified. In each of the 12 conditions shown, the NGF-treated cells showed greater NTBI uptake than the control cells (Figures 3A–3D). Replicating each of these experiments four to six times gave slightly different values of iron uptake but in each experiment the NGF treatment increased the uptake.

Figure 3 NTBI uptake at 0.1, 1 and 10 μM iron in NGF-treated and control cells

Values are means $\pm$ S.E.M. for six replicates. For each histogram pair, the values for the NGF-treated cells (open columns) were significantly higher than for the control (hatched columns) ($P < 0.05$). Replicating these experiments four to six times for each group gave higher values of the NTBI uptake in the treated cells than in the control cells.

Figure 2 Dependence of NTBI uptake in NGF-treated and control PC12 cells on iron concentration

Uptake was performed for 2 min at 0 $^{\circ}\text{C}$ (∇) or 37 $^{\circ}\text{C}$ (Δ). Ascorbate (1 mM) was included when Fe^{2+} was used. The iron-to-NTA ratio was 1:4 and the iron-to-citrate ratio was 1:100. Results are means for six replicates for the uptake at 37 $^{\circ}\text{C}$ and three replicates for that at 0 $^{\circ}\text{C}$. The difference between values at the two temperatures ($\circ$) was considered to be NTBI uptake. Uptake was determined in NGF-treated cells. Insets show analyses of the NTBI uptake data in Eadie-Hofstee plots (v against $v/[S]$, where v is NTBI uptake and $[S]$ is total iron concentration in μM) and Hill plots [$\log [v/(V_{\text{max}} - v)]$ against $\log [S]$, where $[S]$ is in μM]. In (D) the iron uptake at 10 μM was less than that at 3 μM and hence the highest concentration values were not used for these analyses.

Table 1 Effects of inhibitors on Fe²⁺ uptake in PC12 cells

NTBI uptake in NGF-treated PC12 cells was performed at 0 and 37 °C as in Figure 1 but for only 2 min. Only the differences between the uptakes at 0 and 37 °C are shown. The additives were included in the uptake solution. Nitrendipine was added as a 1 mM solution in ethanol. In each experiment the NTBI uptake values were compared by taking the mean uptake without any additive as 100%. Results are means $\pm$ S.E.M. for experiments on the number of days shown in parentheses. * $P < 0.05$ compared with the control.

Additive	Concentration	NTBI uptake (% of control)
Nitrendipine	1 μ M	77.1 $\pm$ 4.3 (4)
Nitrendipine	10 μ M	76.6 $\pm$ 2.6* (4)
ω -Conotoxin	0.1 μ M	96.2 $\pm$ 5.8 (3)
ω -Conotoxin	0.4 μ M	84.9 $\pm$ 11.8 (3)
ω -Conotoxin	1.6 μ M	77.6 $\pm$ 11.9 (3)
Ethanol	0.1 % (v/v)	97.8 $\pm$ 7.2 (2)
Ethanol	1 % (v/v)	96.4 $\pm$ 5.1 (3)

Figure 4 Effect of pretreatment with thapsigargin on NTBI uptake in NGF-treated PC12 cells

A thapsigargin solution in DMSO was added to the cells at 22–24 °C and after 15 min the cells were diluted 3-fold into an NTBI uptake solution as described in the Experimental section. The final concentrations of thapsigargin were 0 (DMSO), 1 and 10 μ M. Uptake was performed for 2 min. Values are means $\pm$ S.E.M. for nine replicates and are expressed as percentages of the mean value of the uptake without any additives. Thapsigargin pretreatment at either concentration produced a significant increase in the NTBI uptake in this experiment and on replicating it ($P < 0.05$).

NTBI uptake and Ca²⁺ entry pathways in NGF-treated PC12 cells

Table 1 shows the effects of the voltage-operated Ca²⁺-channel inhibitors nitrendipine (an L-type channel inhibitor) and ω -conotoxin (an N-type channel inhibitor). Including 1 or 10 μ M nitrendipine in the NTBI uptake solution inhibited the uptake by 20 % ($P < 0.05$). Although the data with ω -conotoxin suggest a trend, it did not produce a statistically significant inhibition at any of the concentrations used ($P > 0.05$). We examined the effects of the endoplasmic reticulum Ca²⁺ pump inhibitor thapsigargin on NTBI in two protocols. In the first protocol we included 1 μ M thapsigargin in the NTBI uptake solution. In four experiments the NTBI uptake was 99 ± 5 % of the control, indicating that this agent had no effect ($P > 0.05$). Because Ca²⁺ depletion of the endoplasmic reticulum on prolonged inhibition of the pump by thapsigargin might produce a different result, we used a second protocol. We preincubated the cells with thapsigargin for 15 min at 22–24 °C and then added them to the NTBI uptake solution (Figure 4). The preincubation with thapsigargin increased NTBI uptake by 20–30 % ($P < 0.05$), with 1 and 10 μ M thapsigargin producing similar effects (Figure 4).

Figure 5 TBI uptake

(A) Time course of TBI uptake by NGF-treated PC12 cells. Cells were incubated with 100 nM ⁵⁵Fe-holotransferrin and filtered after the specified times. Blank values with a 1000-fold excess of unlabelled holotransferrin were subtracted. (B) TBI uptake for 90 min at 10 and 100 nM ⁵⁵Fe-holotransferrin by NGF-treated (open columns) and control (hatched columns) cells. Values are means $\pm$ S.E.M. for six replicates. Replicating the experiment gave similar values for TBI uptake, with the values for the treated cells being higher than those for the control cells.

Figure 6 Western blots showing nNOS expression in NGF-treated and control PC12 cells

(A) Gel showing nNOS bands for 160 kDa standard supplied by the manufacturer (S), 75 g supernatant protein of control (C) and NGF-treated (N) PC12 cells. (B) Relative absorbances. The absorbances of the control and the NGF-treated band were determined and corrected for the background. The relative absorbance of the NGF-treated band was calculated by taking the value for the control band as 100. Results are means $\pm$ S.E.M. for three experiments.

TBI uptake

Figure 5(A) shows a time course of TBI uptake by NGF-treated cells with the use of 100 nM holotransferrin (200 nM Fe³⁺) at 37 °C. The uptake at 0 °C was less than 10 % of the total uptake at 37 °C and could not be monitored reliably because it was too low. In comparison with the NTBI uptake (50 nmol/min per g), the TBI uptake at 37 °C was very slow, with an initial rate of 3–4 nmol/h per g of protein, i.e. 0.05 nmol/min per g. Holotransferrin binding has been reported to have a K_d of 40 nM [2,3]. We compared TBI uptake in NGF-treated and control cells at 37 °C over 90 min at 10 and 100 nM holotransferrin. At either

concentration, TBI uptake was greater in the NGF-treated cells than in the control. However, the rate of TBI uptake was low in both the treated and the control cells (Figure 5B).

nNOS in NGF-treated PC12 cells

Figure 6 shows Western blots (Figure 6A) and quantification (Figure 6B) of nNOS in NGF-treated and control PC12 cells. The nNOS levels were higher in the NGF-treated cells than in the control ($P < 0.05$).

DISCUSSION

We have shown that treatment of PC12 cells with NGF increases NTBI and TBI uptake, although NTBI uptake predominates in these cells. The treatment also accompanies an increase in expression of nNOS. Here we discuss the methods used, possible pathways for NTBI uptake, their possible link to NO and potential implications of these results.

We compared the effects of NGF on PC12 cells by using NTA and citrate to confirm all of our observations. We started out by using conditional stability constants for NTA and Fe^{2+} [23]. However, such constants are available only for the ligand pair Fe^{2+} -NTA and hence we used the method commonly employed in the literature: the use of a molar ratio of 4 NTA to 1 iron or 100 citrate to 1 iron. NTA has been used to chelate iron in several studies because it binds iron with a high affinity but several workers have criticized its use as non-physiological [2,9–11]. However, others prefer to use NTA because citrate has a lower affinity for iron and can form large polymers [24]. In the range of iron and chelator concentrations used we found that the treatment of PC12 cells with NGF resulted in an increase in NTBI uptake independently of the chelator used and whether Fe^{2+} or Fe^{3+} was examined; for example, NGF treatment increased the uptake under each of the 12 different conditions (Figure 3). The observed uptake represents transport across the cell membranes because (1) it is dependent on temperature, (2) EGTA used in the wash solutions would remove any external bound iron, and (3) bursting the cells by hypotonic shock after the uptake in the presence of NTA resulted in the loss of most of the sequestered iron (results not shown).

The main NTBI pathway identified so far is the nramp2 proteins (nramp 1 in macrophages). We detected nramp2 mRNA in the PC12 cells by reverse-transcriptase-mediated PCR (results not shown). However, the NTBI uptake in PC12 cells cannot be explained fully by nramp2 for the following reason. (1) nramp2 transports Fe^{2+} better than Fe^{3+} but our results do not suggest a very large dependence on the oxidation state of iron. Of course, one cannot rule out a link between nramp2 and a reductase in the membrane to allow the Fe^{3+} transport through this pathway. (2) At least some iron entry would need to involve voltage-operated Ca^{2+} channels because the NTBI uptake is partly inhibited by nitrendipine and possibly ω -conotoxin. An inhibition of NTBI uptake by high concentrations of the dihydropyridine nifedipine has also been reported in other cell types [2]. (3) The higher NTBI uptake in cells preincubated with thapsigargin also suggests the possibility that iron might enter cells through the receptor-operated or store-depletion-dependent Ca^{2+} channels. The indication that iron might enter via these Ca^{2+} channels is novel but not surprising because Mn^{2+} is known to enter through this pathway [25]. Iron entry through the receptor-activated or store-depletion-dependent Ca^{2+} channels might be important in neuronal toxicity. However, information should be interpreted with caution until alternative supporting data become available. The maximum value of the TBI uptake is much lower than for the NTBI uptake, suggesting that TBI and NTBI uptake fill

cellular pools that are saturated differently. This is consistent with the previous suggestion that TBI and NTBI transports load different intracellular iron pools. Thus iron seems to enter these cells via multiple pathways.

It is attractive to link the increase in iron uptake directly with the change in the PC12 cells from proliferating to neuronal, because the treatment with NGF alters the proliferating PC12 cells into a neuronal phenotype with conspicuous neurite outgrowth. However, one cannot exclude the possibility that the increase in the iron type is associated with a change in the expression of nNOS rather than a differentiation to neuronal phenotype. NO has been shown to be important in the expression of transferrin receptors and ferritin in macrophages. Iron transport and NO in substantia nigra have also been associated with Parkinson's disease [26,27]. PC12 cells are used as a model for dopaminergic neurons. Therefore iron transport in the neuronal phenotype of PC12 cells, its possible association with NO levels and its increase with receptor-operated Ca^{2+} entry would be important in understanding the aetiology and management of this disease.

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