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Research Paper

Highly Efficient Transfection of Rat Cortical Neurons Using Carbosilane Dendrimers Unveils a Neuroprotective Role for HIF-1α in Early Chemical Hypoxia-Mediated Neurotoxicity

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Purpose. To study the effect of a non-viral vector (carbosilane dendrimer) to efficiently deliver small interfering RNA to postmitotic neurons to study the function of hypoxia-inducible factor-1α (HIF1-α) during chemical hypoxia-mediated neurotoxicity.
Methods. Chemical hypoxia was induced in primary rat cortical neurons by exposure to $CoCl_2$. HIF1-α levels were determined by Western Blot and toxicity was evaluated by both MTT and LDH assays. Neurons were incubated with dendriplexes containing anti-HIF1-α siRNA and both uptake and HIF1-α knockdown efficiency were evaluated.
Results. We report that a non-viral vector (carbosilane dendrimer) can deliver specific siRNA to neurons and selectively block HIF1-α synthesis with similar efficiency to that achieved by viral vectors. Using this method, we have found that this transcription factor plays a neuroprotective role during the early phase of chemical hypoxia-mediated neurotoxicity.
Conclusion. This work represents a proof-of-concept for the use of carbosilane dendrimers to deliver specific siRNA to postmitotic neurons to block selected protein synthesis. This indicates that this type of vector is a good alternative to viral vectors to achieve very high transfection levels in neurons. This also suggests that carbosilane dendrimers might be very useful for gene therapy.

KEY WORDS: dendrimers; HIF1-α; Hypoxia; neurons; neurotoxicity.

INTRODUCTION

Changes in oxygen bioavailability are relevant to central nervous system pathology including certain disorders such as stroke, head trauma and neurodegenerative diseases (1). Changes in O_2 concentration are detected by complex oxygen-sensing systems that elicit a set of adaptative responses both in the short term (minutes) and long term (days) (2). These adaptative responses include decreasing neuronal excitability, suppression of gene transcription and protein synthesis (3,4) and activation of specific transcription factors such as nuclear factor κB (NF-κB), activator protein-1 (AP-1) and hypoxia inducible factor (HIF). Of these factors, HIF plays a pivotal role in regulating adaptative responses to hypoxia (5).

Under normoxic conditions, the transcription factor HIF-1α is continuously synthesized and its protein levels and transcriptional activity are finely regulated by different mechanisms (6). The most important regulatory system includes its proteolysis by different oxygen-dependent mechanisms including hydroxylation by prolyl-hydroxylases (7) that allows its ubiquitination and proteosomal degradation (8). However, under hypoxia or in the presence of different compounds used as chemical models for hypoxia such as $CoCl_2$ (9), prolyl-hydroxylases are inhibited and HIF-1α accumulates in cytosol (9). After dimerization, with constitutively expressed HIF-1β subunit, it translocates to the nucleus where it acts as a transcription factor for more than 60 different proteins that are relevant for processes such as angiogenesis, erithropoiesis and energy metabolism. In addition, the HIF system induces expression of anti-apoptotic proteins like Epo or VEGF (10) and also pro-apototic

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ABBREVIATIONS: 2G-NN16, second generation carbosilane dendrimer; AP-1, activator protein 1; ECL, enhanced chemiluminiscence system; HIF, Hypoxia-inducible factor; LDH, lactate dehydrogenase; MTT, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, Nuclear factor kappa B; PAGE-SDS, poliacrylamide gel electrophoresis; PAMAM, poliamidoamine; PMSF, phenyl methyl sulfonyl fluoride; scRNA, scramble siRNA; SDS, sodium dodecyl sulphate; VEGF, vascular endothelial growth factor.

proteins such as DEC (defective chorion)-1 which causes cell cycle arrest. HIF also stabilizes p53 (11). This dual action makes it difficult to clearly establish if HIF-1α plays a neuroprotective or pro-apoptotic role during hypoxia in the nervous system. One approach to this problem is to perform lack-of-function studies on HIF-1α. Mice deficient in HIF-1α suffer from failure in brain development with extensive cell death in the cephalic mesenchyme (12). However, while HIF-1α relevance to brain development has been clearly established using this model, the lethal phenotype at embryonic day 11 has prevented the study of the role that HIF-1α plays in hypoxia-mediated neuronal death. In order to determine this role, a lack-of function study for this protein in isolated neurons is required.

Small interfering RNA (siRNA) is becoming a very popular method to acutely and selectively knockdown specific proteins. This method allows the role of a specified protein in different physiological and pathological functions to be studied (13). However, the general use of siRNA technology in postmitotic neuronal cells has mainly relied on the use of viral vectors with neuronal tropism. The transfection efficiency of these vectors is estimated to range from 70% to 90% for either adenoviral (14), adeno-associated (15) or lentiviral vectors (16). However, viral vectors have several drawbacks such as the complexity of preparation and possible immune and inflammatory responses described for adenoviruses when used in animals including humans (14,17). These drawbacks have led to the search for new methods, based in non-viral vectors, for safe and efficient delivery of genetic material to postmitotic neurons. Non-viral vectors have shown low transfection efficiency ranging from 0.01% for calcium phosphate co-precipitation (18) to about 25% using Lipofectamine 2000 (19).

Dendrimers are chemical structures of nanometric size than are able to readily cross cell membranes (20) and have been used to deliver siRNA into different cell types (21). Recently, a report using an arginine-grafted PAMAM dendrimer of generation 4 showed a transfection efficiency of 40% (22). However, these reported transfection efficiencies are far from those required to perform lack-of-function studies in neurons. We have recently described the use of water soluble carbosilane-based dendrimers as biocompatible molecules with good perspectives as non-viral vectors for nucleic material (23).

Here, we report that carbosilane dendrimers are efficient and non-toxic vehicles in transfecting primary neuronal cultures achieving more than 80% reduction in protein expression. This efficiency is similar to that obtained using viral vectors. This is the first time that such a decrease in protein levels has been reported for siRNA transfection using non-viral vectors in nervous system cells. We have used this new method to selectively block HIF-1α expression showing that this protein contributes to neuronal survival during the initial hours following cobalt exposure, a treatment that mimics hypoxia.

MATERIALS AND METHODS

Animal Handling

All animals were treated and sacrificed in accordance with guidelines of the European Union (86/609/EEC) for the use of laboratory animals.

Cell Culture

Primary neuronal cultures of brain cortical neurons were essentially prepared as described previously (24). The frontolateral cortical lobes were dissected out of Sprague–Dawley embryonic day 17 fetuses and were mechanically dissociated in Hanks' balanced solution. The cortical lobes were triturated by aspirating seven to ten times using a Pasteur pipette with a flame-narrowed tip. After centrifugation at 800×*g* for 5 min, cells were resuspended in serum-free Neurobasal medium supplemented with B27 containing 2 mM L-glutamine, penicillin (20 U/ml) and streptomycin (5 μg/ml) and plated on poly-L-lysine-coated six-well culture plates or on poly-L-lysine-coated glass coverslips. The combination of Neurobasal, B27 and the lack of serum minimized glial proliferation and the culture contained around 95% pure neurons when stained with the specific neuronal antigen NeuN.

Dendrimer Synthesis and Characterization

Dendrimer 2G-NN16 is a second generation carbosilane dendrimer containing 16 ammonium groups at its periphery and was synthesized as previously described (23). A large excess of MeI (0.06 mL, 0.91 mmol) was added to a solution of 2G-[Si{$O(CH_2)_2N(Me)(CH_2)_2NMe_2$}]$_8$ (0.11 g, 0.04 mmol) in diethylether. The resulting solution was stirred for 48 h at room temperature and then evaporated under reduced pressure to remove residual MeI. The residue was washed with Et_2O (2×5 mL) and dried under vacuum to give 2G-[Si{$O(CH_2)_2N^+(Me)_2(CH_2)_2N^+Me_3$}]$_8$ called 2G-NN16 as a white solid (0.18 g, 86%). The dendrimer chemical structure is shown in Fig. 1. The data on optimal siRNA phosphate dendrimer nitrogen ratio (P/N), polyanion competition and dendrimer-mediated siRNA protection from RNAase degradation have already been published (23).

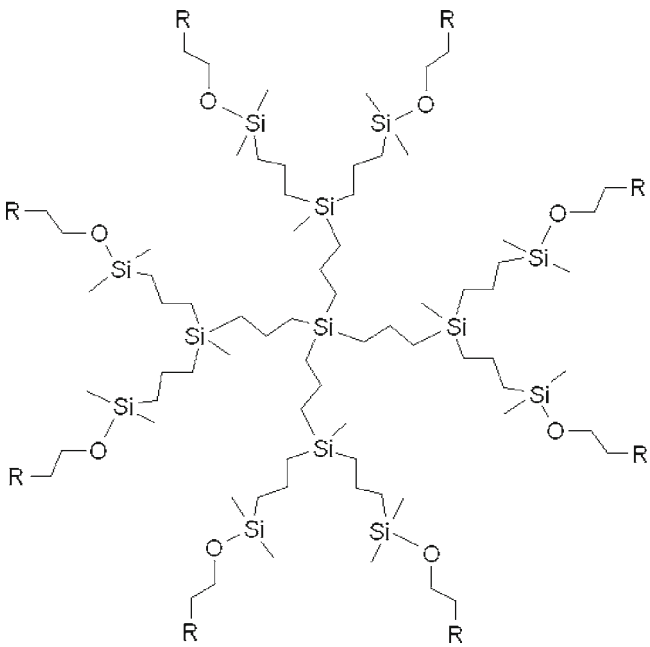

Fig. 1. Chemical structure of 2G-NN16 carbosilane dendrimer. *R* N + (CH3)2CH2CH2N + (CH3)3.

Transfection Efficiency Assays

For transfection assays, cortical neurons were seeded on poly-L-lysine-coated glass coverslips (20 mm) at a density of 2.5×10^5 or 5×10^5 cells/dish. Transfections were performed using 2G-NN16 dendrimers as genetic material carriers. Both non-labelled and fluorescein-labelled HIF-1α siRNA were obtained from Ambion (Cambridgeshire, UK). The siRNA sequence used was 5′-CCAGUUGAAUCUUCAGAUAtt-3′. Fluorescein-labelled siRNA (100 nM)/2G-NN16 complexes were prepared at the optimal P/N ratio of 1/4 (23) in serum-free medium and incubated for 30 min at room temperature. The siRNA/dendrimer complex was added to each dish and cells were then incubated in serum-containing medium for 3, 6 and 18 h at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO_2. Afterwards, cells were washed with PBS and examined using a Leica DMRXA microscope with the appropriate fluorescence filters (excitation wavelength of 490 nm and emission wavelength of 520 nm). Photomicroscopy was performed using a Leica DC500 camera. Transfection efficiency was calculated by counting the number of fluorescein-positive cells over total number of cells in 9 randomly selected regions from three independent experiments.

Toxicity Assays

LDH and MTT assays were performed as previously described (25).

LDH Assay

Cortical neurons were seeded onto 24-well plates at 15×10^4 cells/well and cultured for 6 DIV. After this period of time, neurons were treated with either 2G-NN16 dendrimer (0.5 to 5 μM) for 24 h or with scrambled siRNA/2G-NN16 dendrimer complex for 24–72 h. After incubation periods, supernatants were collected and intact cells were lysed using 0.1% (*w*/*v*) Triton X-100 in (0.9%) NaCl. Both LDH released to cell culture media by neurons, as well as LDH content in the cells were determined spectrophotometrically at 490 nm on a 96-well plate reader using the CytoTox 96 Kit (Promega) following manufacturer's instructions. The percentage of LDH released is defined by the ratio LDH released/total LDH present in the cell at the beginning of treatment. All samples were run in triplicate.

MTT Assay

Cortical neurons were seeded onto 24-well plates at 15×10^4 cells/well, cultured for 6 DIV, and then treated with either vehicle or 200 μM $CoCl_2$ for different periods of time in the presence or absence of caspase 3 inhibitor Z-D(OMe)QMD(OMe)-FMK (IC3). In another set of experiments cells were previously treated with 100 nM scrambled siRNA/dendrimer complex or 100 nM HIF-1α siRNA/dendrimer complex for 18 h. Then, the medium was removed and the cells were incubated with vehicle or 200 μM $CoCl_2$ for 24–72 h.

After the incubation periods, MTT (5 mg/ml) was added to each well (10% total volume), and the cells were incubated at 37°C for 3 h. Next, culture medium was removed and the insoluble formazan crystals were dissolved in 300 μl DMSO (Merck). Aliquots (50 μl) from each well were then transferred to a 96-well microplate, diluted with 150 μl DMSO and measured spectrophotometrically in an ELISA reader (Microplate Reader 2001, Bio-Whittaker) at reference wavelengths of 570 and 630 nm.

Hoescht 33342 Staining

Cortical neurons were seeded on poly-L-lysine-coated glass coverslips (20 mm) at a density of 5×10^5 cells/dish and, after 6 DIV cells, were treated with vehicle or 200 μM $CoCl_2$ for 18–24 h. After the incubation period, cells were loaded with 1 μM Hoescht 33342 for 5 min at 37°C in Krebs–Henseleit solution. Cells were then washed twice with Krebs–Henseleit solution and the fluorescence was observed by using a Leica DMRXA microscope with the appropriate fluorescence filters (excitation wavelength of 350 nm and emission wavelength of 450 nm). Photomicroscopy was performed using a Leica DC500 camera.

Caspase 3 Activity

Cells were plated on poly-L-lysine-coated 6-well culture plates. After 6 DIV, neurons were treated with vehicle (DMSO 1%) or 200 μM $CoCl_2$ for 4–72 h. Afterwards, neurons were washed twice with cold PBS and lysed in Lysis buffer containing 100 mM Hepes pH 7.4, 5 mM DTT, 5 mM EGTA, 0.04% Nonidet P-40, and 20% glycerol. Caspase 3 activity was determined as previously described (25). Extracts were then centrifuged at 5,000×*g* for 10 min at 4°C. Cell extracts (30 μg of protein) were incubated (37°C, 1 h) in reaction buffer (25 mM Hepes, 10% sucrose, 0.1% CHAPS, 10 mM DTT) containing fluorescence substrate Z-DEVD-AFC (50 μM). Cleavage of the AFC fluorophore was determined in a spectrofluorometer at an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Caspase 3 activity was expressed as units of fluorescence × $(\text{mg of protein}\times\text{h})^{-1}$.

HIF-1α Induction

Cortical neurons were plated in poly-L-lysine-coated 6-well culture plates and, after 6 DIV, cells were treated with vehicle or 200 μM $CoCl_2$ for 0.5–24 h. Afterwards, nuclear and cytosolic extracts from neurons were prepared as described (26) and analyzed by gel electrophoresis.

In another set of experiments, cells were pre-incubated with siRNA alone, or 100 nM HIF-1α siRNA/2G-NN16 (1/4) for 18 h. Afterwards, medium was replaced with fresh medium containing vehicle or 200 μM $CoCl_2$. Four hours later, medium was removed and cells were washed twice with cold PBS and resuspended in homogenization buffer (10 mM HEPES, 0.32 M sucrose, 100 μM EDTA, 1 mM DTT, 0.1 mM PMSF, 40 μg/ml aprotinine, 20 μg/ml leupeptine; pH 7.4). Cells were homogenated using a polytron (two cycles, 10 s at maximum speed). Homogenates were then centrifuged at 5,000×*g* for 10 min at 4°C. The supernatants, i.e. total lysates, were removed and analysed by gel electrophoresis.

Western Blot Analysis

Protein samples from nuclear and cytosolic extracts and total lysates (20 μg) were loaded on 10% PAGE-SDS gels and transferred onto nitrocellulose membranes. Membranes were blocked in PBS-Tween 20 (0.1%) containing 5% non-fat dry milk and 0.1% BSA for 1 h at 4°C and incubated with anti-HIF-1α monoclonal antibody (1:5,000) (RD System, Abingdon, England), α-tubulin polyclonal antibody (1:2,000) (Calbiochem, Barcelona, Spain) or Histone H2A polyclonal antibody (1:1,000) (Cell Signaling, Beverly, MA) overnight at 4°C. Afterwards, blots were washed with PBS-Tween 20 (0.1%) and incubated with Horse Rabbit Peroxidase conjugated (HRP)-anti-mouse IgG (1:10,000) for 2 h at 4°C. Immunoreactive bands were visualized using an enhanced chemiluminiscence system (ECL; Amersham Biosciences). Densitometric analysis of immunoreactive bands was performed by using ImageQuant 5.2 software (Amersham Biosciences) and results were expressed as the ratio HIF-1α optical density (O.D. HIF-1α)/α-tubulin optical density (O.D. α-tubulin) for total lysates and cytosolic fractions and as the ratio O.D. HIF-1α/ optical density for Histone H2A (O.D. H2A) for nuclear fractions.

RESULTS

Cobalt Causes Apoptotic Death of Rat Cortical Neurons by Activating Caspase 3

Cobalt treatment is considered a good model for hypoxia because it reproduces one of the key elements of hypoxia: an increase in HIF-1α levels produced by the blockade of prolyl hydroxylases by the cobalt ion (9). Treatment of rat cortical neurons in culture with $CoCl_2$ (200 μM) for 24 h caused neuronal death that showed hallmarks of apoptotic death such as chromatin condensation and fragmentation which could be seen when the cells were stained with the dye Hoechst-33342 (Fig. 2). Mitochondrial damage was studied using MTT assay. As can be observed in Fig. 3a, MTT activity was markedly decreased by cobalt treatment. The inhibition increased with time from about 80% that of control values after 18 h of treatment to about 50% at 48 h (Fig. 3a). It is important to note that lactate dehydrogenase (LDH) activity cannot be used as an index of cell death when HIF-1α is induced, because the expression of this enzyme is upregulated by HIF-1α. Further corroborating the results of mitochondrial damage caused by cobalt, the ion also induced an increase in caspase-3 activity in cortical neurons that was evident after 6 h of treatment, peaked at 36 h and remained high for more than 60 h (Fig. 3b). According to the idea that caspase-3 is the effector caspase in apoptosis, the presence of a caspase-3 inhibitor (IC3) completely prevented cobalt-mediated mitochondrial toxicity (Fig. 3c).

Cobalt Induces an Increase in HIF-1α Protein Levels

It is generally accepted that cobalt mimics hypoxia by increasing HIF-1α protein levels (9). As can be observed in Fig. 4a, treatment of cortical neurons in culture with $CoCl_2$ (200 μM) induced an early increase in HIF-1α levels that was evident after 2 h with a peak at 6 h in the cytosol (Fig. 4a) and after 12 h with a peak at 4 h in the nuclei (Fig. 4b).

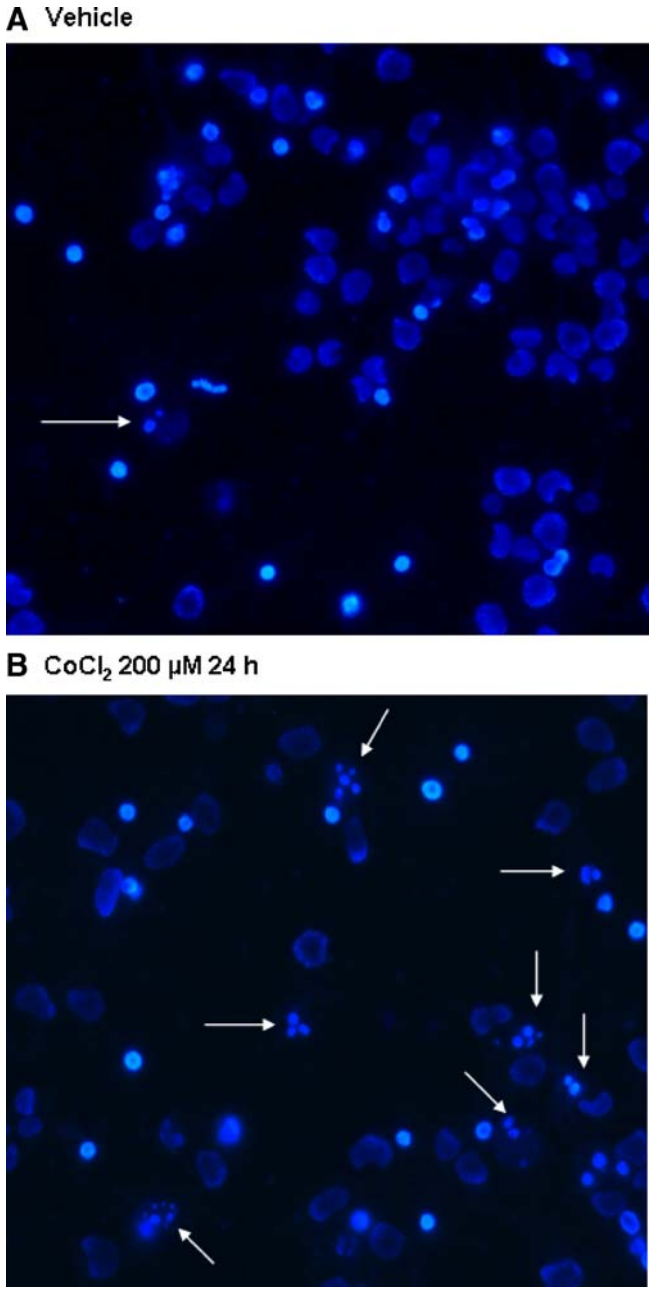

Fig. 2. Morphological characterization of $CoCl_2$-induced cortical neuron death. **A** Hoechst staining of cortical neuron nuclei treated with vehicle. Cells were loaded with 1 μM Hoescht 33342 in Krebs–Henseleit solution for 5 min at 37°C. **B** Same as in **A**, but neurons were treated with 200 μM $CoCl_2$ for 24 h.

Dendrimers Lack Toxicity in Neurons

To exclude the possibility of toxicity caused by the carbosilane dendrimer 2G-NN16 alone on rat cortical neurons, cells were exposed to 2G-NN16 dendrimer at concentrations ranging from 0.5 to 5 μM for 24 h. Toxicity was

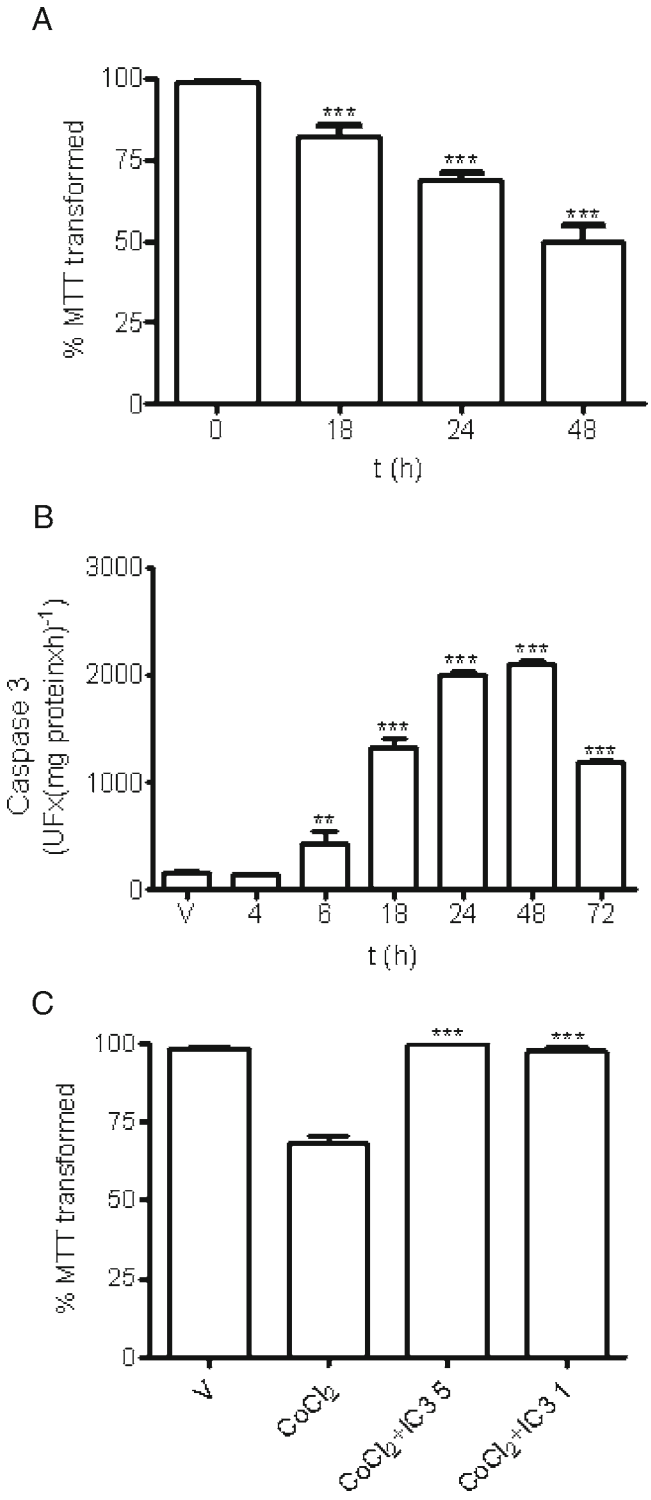

Fig. 3. Role of caspase 3 in $CoCl_2$-mediated neuronal death. **A** Time-course of cortical neuron viability in the presence of 200 μM $CoCl_2$ expressed as percentage of MTT transformed related to vehicle-treated cells. Data are expressed as mean±SEM, $n=12$. ***$P<0.001$, as compared with vehicle-treated cells. **B** Time-course of caspase-3 activation induced by 200 μM $CoCl_2$ in rat cortical neurons. Data are expressed as mean±SEM, $n=12$. ***$P<0.001$, as compared with vehicle-treated cells. **C** Cortical neurons were incubated with vehicle or 200 μM $CoCl_2$ in the absence or presence of caspase-3 inhibitor (*IC3*) for 24 h and cellular viability expressed as percentage of MTT transformed related to vehicle-treated cells was determined. Data are expressed as mean±SEM, $n=12$. ***$P<0.001$, as compared with vehicle-treated cells.

measured using the MTT assay. As can be observed in Fig. 3a, for up to 5 μM dendrimer concentration, MTT activity was above 95% that of control values. This suggested that, at these concentrations, the 2G-NN16 dendrimer lacked significant toxicity (Fig. 5a). A similar result was obtained when LDH release was used as an indicator of toxicity (Fig. 5b) indicating a good correlation between both methods at estimating cell damage. In addition, treatment with dextran (0.5 to 5 μM) lacked toxic effects (data not shown) excluding the possibility that the toxic effects were due to changes in osmolarity. Neurons were also transfected with a complex containing 2G-NN16 dendrimer and a scrambled siRNA containing the same nucleotides as the specific siRNA, but randomly mixed and lacking biological function, (scRNA/2G-NN16) as a control for HIF-1α siRNA. As can be observed in Fig. 5c, d, no toxicity was observed for up to 72 h for a scRNA/2G-NN16 complex (100 nM). These results suggest that neither the 2G-NN16 dendrimer alone nor the dendrimer-scRNA dendriplex are toxic. It is important to note that in this set of data, LDH could be used as a toxicity index because no HIF-1α induction took place.

Transfection Efficiency in Primary Cortical Neuron Cells

To optimize transfection efficiency as a function of incubation time, neurons were incubated with fluorescein-labelled siRNA alone or with fluorescein-labelled siRNA/2G-NN16 complex at a P/N ratio of 1/4 for 3, 6 and 18 h and then transfection efficiency was measured 24 h later. Neurons treated with fluorescein-labelled siRNA alone showed very scarce fluorescein-positive cells (less than 2%) (Fig. 6a; top panel). However, incubations of neurons in the presence of the complex HIF-1α siRNA/2G-NN16 dendrimer for different times showed an increase in the number of fluorescein-positive neurons with time, that amounted to 16.83±5.84% at 3 h, 64.34±17.09% at 6 h and 87.40±5.05% at 18 h of incubation of the total number of cells present in the culture (Fig. 6a, bottom panel).

Down-regulation of HIF-1α Using siRNA/2G-NN16 Complex in Primary Cortical Neuron Cultures

To knock-down HIF-1α protein levels, primary cortical neurons were incubated with either vehicle, HIF-1α siRNA (100 nM) alone, dendriplex scRNA/2G-NN16 (100 nM) or dendriplex HIF-1α siRNA/2G-NN16 (100 nM) for 18 h to allow internalization of the nucleic material. Next, medium was removed and the cells were treated with either vehicle or $CoCl_2$ (200 μM) for 4 h. Western blot analysis of total cellular lysates showed that untreated cortical neuronal cells did not express significant HIF-1α protein levels (Fig. 6b and c) whereas $CoCl_2$ induced about a 10-fold increase in HIF-1α/α-tubulin ratio (Fig. 6b, c). Treatment of cortical neurons with siRNA alone or with scRNA/2G-NN16 complex did not modify $CoCl_2$-induced HIF-1α levels (Fig. 6b,c). However,

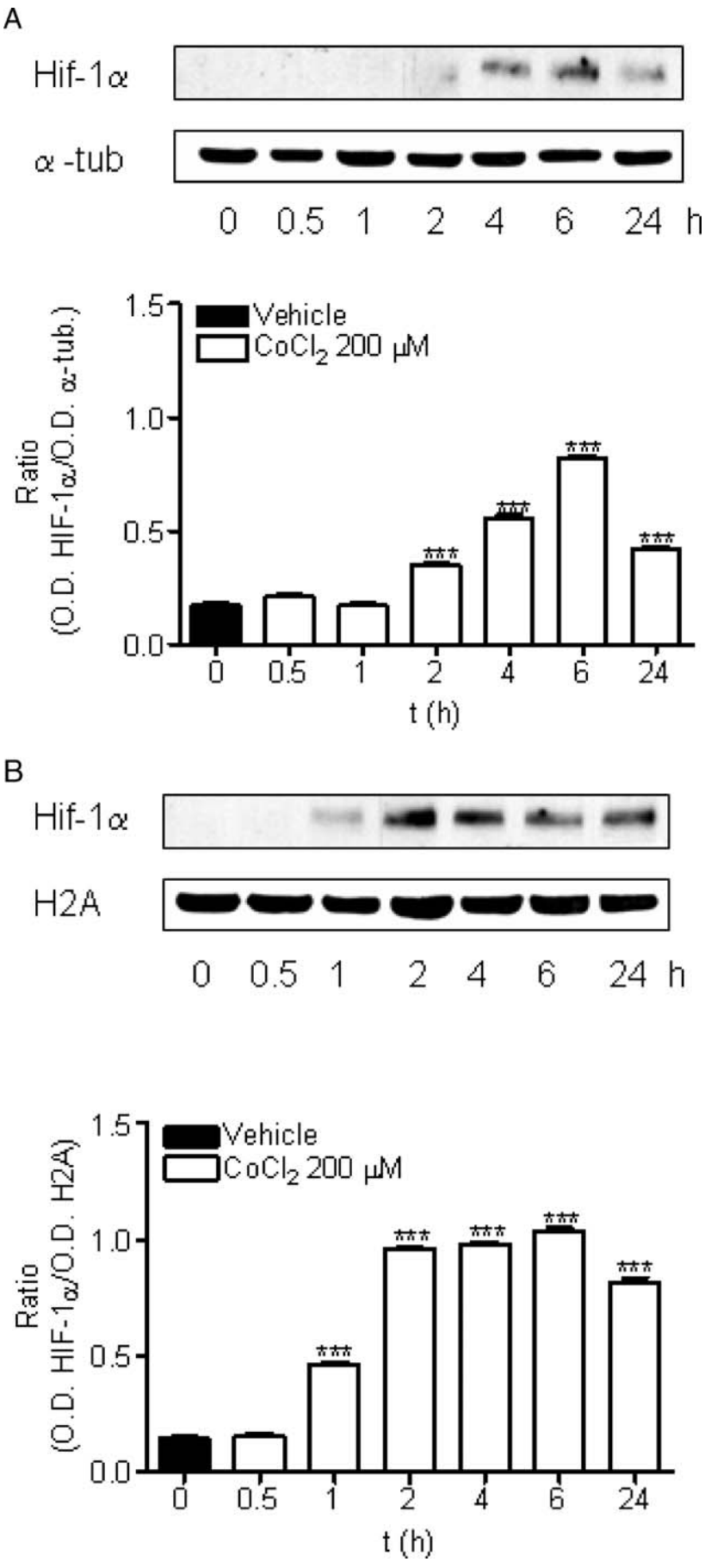
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Fig. 4. $CoCl_2$ induces HIF-1α protein stabilization and translocation to the nucleus. Cytosolic and nuclear fractions were obtained form cortical neurons treated either with vehicle or 200 μM $CoCl_2$ for different periods of time and HIF-1α expression was studied. **A** *Top panel.* Time-course of HIF-1α expression observed in cytosolic fractions obtained from cortical neurons treated with 200 μM $CoCl_2$ for different times. α-tubulin (*α-tub.*) was used as cytosolic protein loading control. *Bottom panel.* Densitometric analysis of the results. **B** *Top panel.* Time-course of HIF-1α expression observed in nuclear fractions obtained from cortical neurons treated with 200 μM $CoCl_2$ for different times. Histone 2A (*H2A*) was used as nuclear protein loading control. *Bottom panel.* Densitometric analysis of the results.

treatment of the neurons with HIF-1α siRNA/2G-NN16 complex for 18 h prior to $CoCl_2$ exposure, markedly reduced $CoCl_2$-induced HIF-1α expression (Fig. 6b, c). Therefore the method was very efficient at specifically decreasing protein expression in neural tissue. In addition, these data show that siRNA is efficiently internalized into the neurons where it blocks HIF-1α protein synthesis.

HIF-1α Prevents Cobalt-induced Toxicity in Neurons

HIF-1α protein knockdown using the dendriplex HIF-1α siRNA/2G-NN16 markedly increased early cobalt-induced toxicity in rat cortical neurons (Fig. 7). Cobalt-mediated toxicity in the absence of high HIF-1α levels at 1 h was similar to that obtained after 24 h of treatment in control neurons. This enhancement of toxicity was maintained up to 6 h and then disappeared after 24 h of treatment (Fig. 5). The HIF-1α siRNA effect in cobalt-treated cells was sequence-specific because no effect could be observed for the dendriplex scRNA/2G-NN16. This suggests that HIF-1α might contribute to neuron survival during early cobalt-induced toxicity. It is important to note that caspase-3 activation in response to cobalt starts to rise at 6 h and is evident at 10 h after cobalt treatment.

DISCUSSION

The study of the role of different proteins in neuronal physiology or pathology requires an approach that should include the selective knockdown of such proteins to study a lack-of-function effect. One traditional approach is to generate knock-out mice for the selected protein (27), but this is a time-consuming method and, sometimes, the function of the lacking protein can be replaced by another protein during

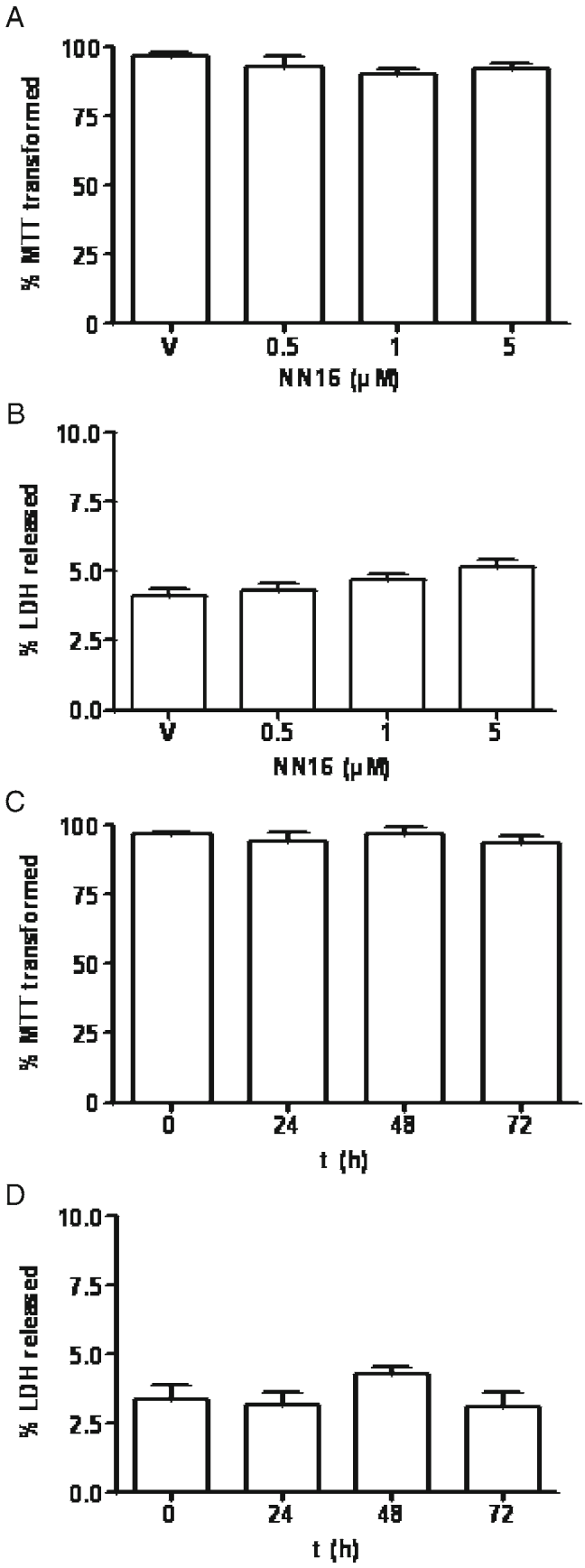

Fig. 5. 2G-NN16 dendrimer and siRNAscrambled/2G-NN16 dendrimer complex lack toxicity on rat cortical neurons. Cells were treated with vehicle or 2G-NN16 (0.5 to 5 μM) for 24 h and neuron viability was assayed by measuring percentage of MTT transformed related to that in vehicle-treated cells (**A**) or by quantifying percentage of LDH released to culture medium (**B**). Data are expressed as mean±SEM, $n=12$. **C** Cortical neurons were treated with either vehicle or 100 nM scrambled siRNA/2G-NN16 dendrimer dendriplex for 24 to 72 h and cellular viability was assayed by measuring either the percentage of MTT transformed related to that in vehicle-treated cells (**C**) or the amount of LDH released to the medium (**D**). Data are expressed as mean±SEM, $n=12$.

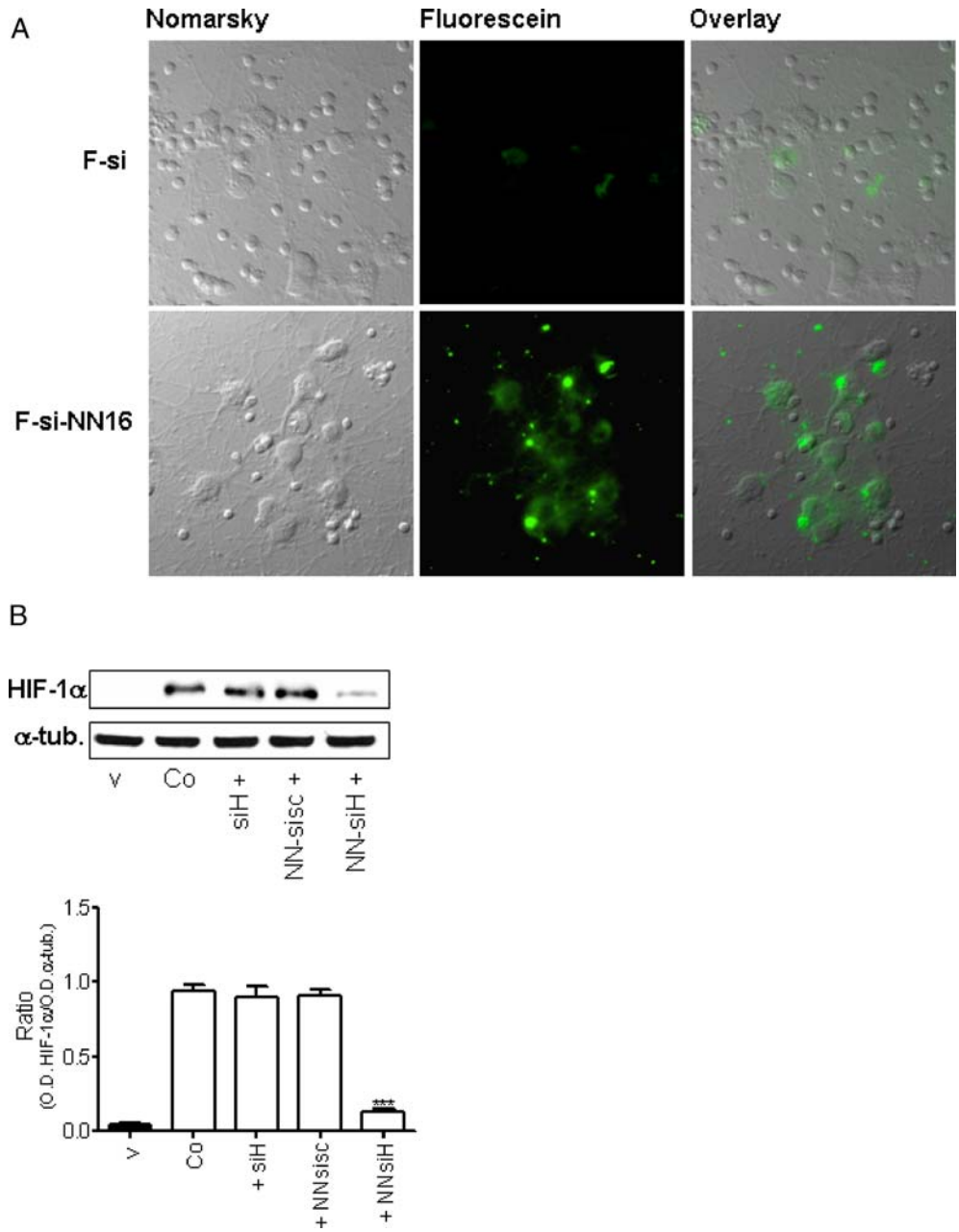

Fig. 6. Uptake and transfection efficiency of 2G-NN16 dendrimer as a delivery vector for siRNA in primary neuron culture. **A** Nomarski and fluorescence images of rat cortical neurons treated with either FAM-labelled HIF-1α siRNA alone (*F-si*) (*top images*) or FAM-labelled HIF-1α siRNA/2G-NN16 dendrimer complex (*F-si-NN16*) (*bottom images*) for 18 h. The overlay between both images is also shown. **B** *Top panel.* HIF-1α expression in rat cortical neurons treated with either vehicle (*v*) or 200 μM $CoCl_2$ (*Co*) for 4 h, alone or after the neurons were pre-incubated, for 18 h, in the presence of HIF-1α siRNA alone (+*siH*), scrambled siRNA/2G-NN16 dendrimer complex (*NNsisc*) or HIF-1α siRNA/2G-NN16 dendrimer complex (*NNsiH*). *Bottom panel.* Densitometric analysis of HIF-1α protein levels expressed as ratio of HIF-1α optical density (O.D. HIF-1α) and α-tubulin optical density (O.D. α-tubulin). Data are expressed as mean±SEM, $n=3$. *** $P<0.001$, as compared with $CoCl_2$-treated cells.

development resulting in no change in phenotype. In addition, the alternate phenotype can be lethal very early in embryonic development preventing further studies as is the case for HIF-1α (12). Another approach is to generate conditional knock-down or knock-in mice that only down-regulate or express the protein with a specific treatment (28). This procedure prevents compensation of function by another protein during development. However, it still is very time consuming.

Small interfering RNA has emerged as a good alternative to knock-out mice for studying the role that certain proteins play in physiological and pathological mechanisms. siRNA is specific, it does not allow for the genesis of compensatory pathways during development, and it is cheaper and less time

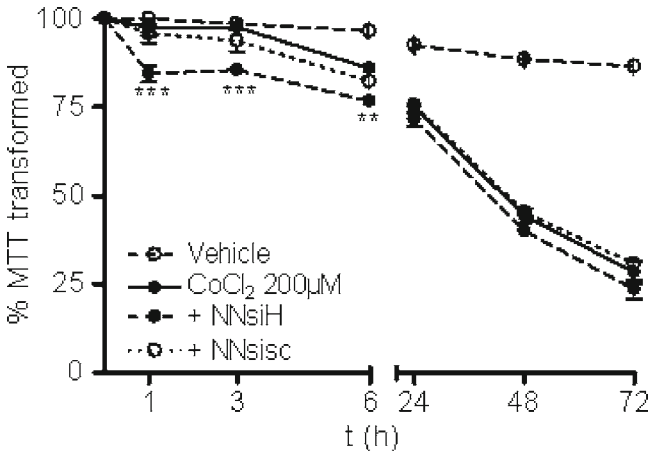

Fig. 7. HIF-1α stabilization results in early protection from $CoCl_2$-induced cortical neuron cytotoxicity. Cortical neurons were pre-incubated with 100 nM scrambled siRNA/2G-NN16 dendrimer complex (*+NNsisc*) or 100 nM HIF-1α siRNA/2G-NN16 dendrimer complex (*+NNsiH*) for 18 h and then incubated with either vehicle or 200 μM $CoCl_2$ for different periods of time. Cortical viability was determined by measuring the percentage of MTT transformed related to vehicle-treated cells. Data are expressed as mean±SEM, $n=12$. ***$P<0.001$ as compared with $CoCl_2$-treated cells.

consuming than knock-out mice generation (13). However, siRNAs must be delivered into the interior of the cell in order to perform its inhibitory function. This can be accomplished using either non-viral (22) or viral (16) vectors. In neural cells, due to the low transfection efficiency of non-viral vectors (Table I), neurotropic viruses have been routinely used to introduce siRNA into neurons and have achieved up to 80% inhibition of specific protein levels (14,16).

Dendrimers are chemical structures of nanometric size than are able to readily cross cell membranes (29) and have been used to deliver siRNA into different cell types (30). Dendrimers have emerged as an alternative approach to liposomes and polymeric systems for drug delivery and nucleic acid transfection. Their major advantages include uniform structure, multiple sites of attachment and the versatility in modifying their skeletons and surfaces, allowing a precise characterization of the dendrimer/drug or nucleic acid interaction. The first study describing the use of dendritic macromolecules for transfection utilized polyamidoamine (PAMAM) dendrimers (31). Since then, extensive studies have been performed despite mostly falling short of the required transfection efficiency to decrease protein levels in neurons to a similar extent as that achieved by viral vectors (Table I).

Here, we show that a carbosilane dendrimer coupled to siRNA has a transfection efficiency of about 85% in rat cortical neurons. This is similar to that achieved using viral vectors (16) and much higher than that obtained with other methods previously described (Table I). None of the methods previously described results in a reduction in protein levels to an extent that would allow the study of a lack-of-function effect. Furthermore, we have seen that 2G-NN16 dendrimer causes very low toxicity in neural cells. The data presented here indicate that this is a new, simple, fast and non-toxic method to efficiently deliver siRNA to neurons.

HIF-1α is a transcription factor that is stabilized and increases in concentration during hypoxia or treatment with $CoCl_2$ (a good model of chemical hypoxia). This upregulation occurs through various mechanisms with the inhibition of its degradation via the proteosomal pathway being the most prominent (8). Cobalt treatment induces cortical neuron death by an intrinsic apoptotic mechanism involving mitochondria. In this mechanism, caspase-3 is activated and functions as the effector caspase (Fig. 3c). Knockdown of HIF-1α protein expression, through the use of a carbosilane dendrimer to efficiently deliver a specific siRNA, shows that HIF-1α, is likely an antagonist to early cobalt-mediated toxicity. This possibly occurs via the expression of some genes that are dependent on this transcription factor. However, after 6 h of cobalt treatment, other mechanisms such as cytochrome-c release from mitochondria, are activated and trigger non-return apoptotic signaling and caspase-3 activation that overcome the neuroprotective action of HIF-1α. It is important to note that the time window during which HIF-1α shows a protective effect lasts only as long as no increase in cobalt-mediated caspase-3 activity can be observed (Fig. 1c). This would suggest that HIF-1α might interfere with the release of pro-apoptotic factors from mitochondria. This is a relevant finding because, so far, the effect of HIF-1α removal on chemical hypoxia-mediated toxicity has not been studied since HIF-1α KO mice have a phenotype that causes its death at embryonic day 11 (12) thus preventing the study of the effect of various treatments on HIF-1α-deficient isolated neurons.

CONCLUSION

The results indicate that 2G-NN16 dendrimer is able to transfect siRNA molecules to the interior of primary neuronal cultures and for the first time achieve protein knockdown at efficiency levels similar to those obtained using viral vectors. This represents a proof-of-concept indicating that 2G-NN16 dendrimer is an easy and efficient alternative to viral vectors to selectively block protein expression in neurons allowing the study of a lack-of-function effect for different

Table I. Transfection Efficiency in Neuronal Cells Achieved by Different Methods

Transfection Vector	Primary culture cells	% Transfection efficiency	Reference
Calcium phosphate	Rat Schwann cells	0.01	(18)
Cationic lipid: Polyethylenimine (BPEI)	Rat hypothalamic neurons	14	(32)
Cationic lipid: Lipofectamine 2000	Rat cortical neurons Rat hippocampal cells	25–27	(19)
4-G dendrimer: PAMAM-Arg	Rat glia and neurons mixed culture	40	(22)
2-G dendrimer: NN16	Rat cortical neurons	80	In this paper
Lentivirus	Mice cerebellar granule neurons	81–92	(16)

proteins in primary neurons. Using this methodology, we have been able to show, for the first time, that HIF-1α plays a neuroprotective role during the early phase of cobalt-mediated neuronal toxicity. Although more experiments are needed to identify the exact mechanism of the HIF-1α protective effect, the fact that cobalt treatment is a generally accepted model of chemical hypoxia, suggests that HIF-1α should act as a neuroprotective molecule during hypoxic insults.

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