Biochimica et Biophysica Acta 1840 (2014) 495–506

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Transcriptional profile of genes involved in oxidative stress and antioxidant defense in PC12 cells following treatment with cerium oxide nanoparticles

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ARTICLE INFO

Article history:
Received 12 August 2013
Received in revised form 23 September 2013
Accepted 7 October 2013
Available online 15 October 2013

Keywords:
PC12 cells
Cerium oxide nanoparticles
Oxidative stress
Anti-oxidant defenses
Neuroprotection

ABSTRACT

Background: Thanks to their impressive catalytic properties, cerium oxide nanoparticles (nanoceria) are able to mimic the activity of superoxide dismutase and of catalase, therefore acting as reactive oxygen species (ROS) scavengers in many biological contexts, for instance offering neuroprotection and reduction of apoptosis rate in many types of cells exposed to oxidative stress (stem cells, endothelial cells, epithelial cells, osteoblasts, *etc.*).
Methods: We report on the investigation at gene level, through quantitative real time RT-PCR, of the effects of cerium oxide nanoparticles on ROS mechanisms in neuron-like PC12 cells. After three days of treatment, transcription of 84 genes involved in antioxidant defense, in ROS metabolism, and coding oxygen transporters is evaluated, and its relevance to central nervous system degenerative diseases is considered.
Results: Experimental evidences reveal intriguing differences in transcriptional profiles of cells treated with cerium oxide nanoparticles with respect to the controls: nanoceria acts as strong exogenous ROS scavenger, modulating transcription of genes involved in natural cell defenses, down-regulating genes involved in inflammatory processes, and up-regulating some genes involved in neuroprotection.
Conclusions: Our findings are extremely promising for future biomedical applications of cerium oxide nanoparticles, further supporting their possible exploitation in the treatment of neurodegenerative diseases.
General significance: This work represents the first documented step to the comprehension of mechanisms underlying the anti-oxidant action of cerium oxide nanoparticles. Our findings allow for a better comprehension of the phenomena of ROS scavenging and neuroprotection at a gene level, suggesting future therapeutic approaches even at a pre-clinical level.

1. Introduction

Cerium oxide nanoparticles (nanoceria, NC) are an oxide of the rare earth metal cerium (CeO_2) that, thanks to their surface oxygen vacancies changing the local electronic and valence arrangement, are able to mimic the activity of superoxide dismutase and of catalase, therefore acting as reactive oxygen species (ROS) scavengers in many biological contexts [1,2]. These vacancies compensate the fraction of Ce in the Ce^{3+} form, implying the coexistence of Ce^{3+} and Ce^{4+} ions that are involved in the redox reactions [3]. Recently, NC has been found to act also as scavenger of nitric oxide radical, highlighting further important implications in biological contexts [4].

All these phenomena are enhanced at the nanoscale level because of the higher surface/volume ratio of the nanoparticles, and have already found many possible exploitations in biomedicine, including endothelial cell protection [5], wound healing [6], anti-cancer applications [7–9], neuroprotection [10,11], and neuronal regeneration [12].

However, the interest towards bio-applications of NC is rapidly growing [13,14], and many other examples of possible exploitation can be found in the literature. It has been shown, for example, as NC positively affects angiogenesis by modulating the intracellular oxygen environment [15], and how it exerts therapeutic effects on retinal pathologies [16–18], protecting the retina through a decrease of ROS levels [19]. Moreover, radio-protective effects of cerium oxide nanoparticles have been efficiently demonstrated [20,21].

Concerning potential neurological applications, we recently investigated NC interactions with PC12 cell line that represents a valuable model for many features of central dopaminergic neurons. We confirmed NC anti-oxidant properties on these cells, and, most interestingly, we highlighted both an increment of differentiation in terms of neurite length, and a strong increment of dopamine secretion following NC treatment under proliferative conditions [22].

While the achieved results are very encouraging as far as NC use for central nervous system treatment is concerned, the mechanisms underpinning these effects are still largely unknown. In this work, we

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http://dx.doi.org/10.1016/j.bbagen.2013.10.009

assessed the nanoparticle internalization by PC12 cells through confocal microscopy, and we performed a detailed analysis at gene level of the effects of NC administration, through quantitative real-time RT-PCR. In particular, we focused on oxidative stress and antioxidant defense, in order to depict a clearer picture of the interactions between NC and cells.

Results showed that, out of 84 investigated genes, 16 were affected by NC treatment, denoting an alteration of the natural anti-oxidant defenses of the cells and some interesting compensation mechanisms due to the presence of exogenous ROS scavengers.

2. Materials and methods

2.1. Cell culture and treatment with nanoceria

Cerium oxide nanoparticles were purchased from Sigma (code 544841) and were extensively characterized in a previous work [22]. Nanoparticles were dispersed through a mild sonication in physiologic saline solution (0.9% NaCl in H_2O) at a concentration of 10 mg/ml, and then diluted in cell culture medium at desired concentrations. The obtained dispersions were characterized with scanning electron microscopy through a Dual-Beam system (FEI Helios 600), by dropping a small quantity of diluted dispersion on a silicon wafer, gold-sputtered before observation. UV/Vis spectrum of NC dispersion was performed in the range of 200–1000 nm (Lambda 45 Spectrophotometer, PerkinElmer).

Derived from a rat pheochromocytoma, PC12 cells (ATCC CRL-1721) represent a valuable model for neuronal differentiation, also mimicking many features of central dopaminergic neurons, dopamine production included [23]. PC12 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% horse serum (HS), 5% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

PC12 cells were cultured at a density of 10,000 cells/cm^2 in 24-well plates, and treated for 72 h with 0, 20 and 50 μg/ml of NC.

2.2. Nanoceria internalization assessment

In order to verify NC internalization by PC12 cells, nanoparticles were labeled with a fluorescent dye (Oregon Green 488, from Invitrogen). The chemical modification of NC was achieved adapting procedures previously described in the literature [24]. Nanoparticles were dispersed in a HNO_3 solution (65% w/w), achieving a final concentration of 10 mg/ml, and sonicated for 3 h with a Bransonic sonicator 2510, using an output power of 20 W. This step aimed at introducing −OH groups on nanoparticle surface through a strong oxidation process. Thereafter, nanoparticles were rinsed three times in absolute ethanol (through centrifugations at 30,000 *g* for 10 min each), re-dispersed at a concentration of 10 mg/ml in a solution of 3-aminopropyl-triethoxysilane (APTES, 50% in ethanol, 440140 from Sigma), and stirred at 60 °C for 12 h. Following this procedure, amino groups were exposed on the nanoparticle surface, and further modifications were allowed. Amino-functionalized NC was finally washed three times with deionized water by ultracentrifugation, freeze-dried overnight and stored at −20 °C for further reactions or characterization. Efficacy of the APTES functionalization was verified through attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy. Analyses were performed with a Shimadzu IRAffinity-1 spectrophotometer (4 cm^{-1} resolution in the range of 4000–400 cm^{-1}), and results are reported in the Supplementary material.

The obtained amino-functionalized nanoparticles were dispersed (10 mg/ml) in 1 ml of a 0.1 M borate buffer, pH 8.2, and 100 μl of Oregon Green 488 (10 mg/ml in DMSO) was added drop by drop while stirring. After 6 h of stirring at room temperature, Oregon Green 488-conjugated NC was washed in buffer by several ultracentrifugation steps to eliminate non-reacted dye. The procedure was repeated until no fluorescence was detectable in the supernatant. The efficiency of reaction was evaluated by measuring dye concentration in the supernatants with a microplate reader (Victor3, PerkinElmer; excitation wavelength 485 nm, emission wavelength 535 nm) and, by difference, calculating the amount of dye bound to the nanoparticles. After the final step, fluorescent NC was freeze-dried overnight. Efficiency of the procedure was assessed to be around 60%.

Fluorescent NC was incubated with PC12 cells for 72 h at a concentration of 20 μg/ml. Thereafter, cells were fixed using a 4% paraformaldehyde solution in PBS and stained with TRITC-phalloidin and DAPI through standard procedures in order to label, respectively, f-actin (in red) and nuclei (in blue). Samples were observed with a Nikon Inverted Microscope TiE, equipped with a Nikon Confocal Laser System C2*s* (software for image analysis is NIS Elements).

2.3. Quantitative RT-PCR

Transcription of specific genes was evaluated with quantitative real-time qRT-PCR after three days of incubation with 0, 20, and 50 μg/ml of NC.

Total RNA was isolated from cell cultures using High Pure RNA Isolation kit (Roche) according to the manufacturer's protocol. Extracted RNA was diluted ten times in pure water (MilliQ Millipore) and RNA concentration was measured at 260 nm with a spectrophotometer (Lambda 45, PerkinElmer). RNA retrotranscription into cDNA was performed with 400 ng of RNA in a total volume of 20 μl, including 4 μl of iScript™ Reverse Transcription Supermix (5×, Bio-Rad). The synthesis program included an initial incubation at 25 °C for 5 min, followed by incubation at 42 °C for 45 min and at 48 °C for 15 min. Reaction was inactivated by heating at 85 °C for 5 min, and the reaction volume was finally increased up to 200 μl with pure water.

cDNA was analyzed with the Rat Oxidative Stress RT^2 Profiler™ PCR Array (PARN-065ZD-2 from Qiagen), that profiles the expression of 84 genes related to oxidative stress, to reactive oxygen species (ROS) metabolism, involved in superoxide metabolism, and to relevant oxygen transporter genes. Quantitative RT-PCR was performed with a RT^2 SYBR Green PCR Master Mix (330500 from Qiagen) in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) to determine the transcription of different genes. The thermal protocol was applied with one incubation of 30 s at 98 °C for enzyme activation, followed by 40 cycles at 98 °C for 3 s and 60 °C for 7 s. After the last reaction cycle, the protocol included a temperature ramp from 65 °C to 95 °C, with 0.5 °C/s increments, to exclude unspecific products with melting curve results. Data were normalized by using multiple housekeeping genes (B2m, Hprt1, Ldha, Rplp1), and analyzed by comparing ΔΔCt (difference between ΔCt values, deriving from difference between cycle threshold value Ct of target and housekeeping genes). Details about the investigated genes are reported in Table 1.

2.4. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test to test for significance that was set at $p < 0.05$, with the aid of KaleidaGraph (Synergy Software) and Bio-Rad CFX Manager (Bio-Rad). In all cases, three independent experiments were carried out. Results are presented as mean value ± standard deviation.

3. Results

An extensive characterization of the cerium oxide nanoparticles used in this study, including transmission electron microscopy, energy-dispersive X-ray spectroscopy, and X-ray photoelectron spectroscopy has been previously reported [22]. Briefly, nanoparticles appear as a powder of relatively dispersed size distribution (5–80 nm), with a cubic crystalline structure, high purity, and a Ce^{3+} content of ~23%,

Table 1
List of investigated genes involved in ROS signaling, with Unigene and GeneBank codes, abbreviation, name, and description.

Unigene	Gene bank	Symbol	Description	Gene Name
Rn.202968	NM_134326	Alb	Albumin	Alb1, Albza
Rn.6408	NM_001013413	Als2	Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)	–
Rn.15681	NM_019363	Aox1	Aldehyde oxidase 1	–
Rn.88057	NM_012499	Apc	Adenomatous polyposis coli	RATAPC
Rn.32351	NM_138828	Apoe	Apolipoprotein E	APOEA
Rn.3001	NM_012520	Cat	Catalase	CS1, Cas1, Cs–1, RATCAT01, RATCATL
Rn.8019	NM_031116	Ccl5	Chemokine (C–C motif) ligand 5	Rantes, Scya5
Rn.12311	NM_053425	Ccs	Copper chaperone for superoxide dismutase	–
Rn.100909	NM_022597	Ctsb	Cathepsin B	–
Rn.5856	NM_024160	Cyba	Cytochrome b–245, alpha polypeptide	p22–phox
Rn.105938	NM_130744	Cygb	Cytoglobin	MGC95105, Staap, Stap
Rn.9470	NM_130744	Dhcr24	24–dehydrocholesterol reductase	–
Rn.11231	NM_013199	Dnm2	Dynamin 2	DYIIAAB
Rn.162682	NM_153739	Duox1	Dual oxidase 1	–
Rn.55542	NM_024141	Duox2	Dual oxidase 2	Thox2
Rn.16016	NM_001024897	Ehd2	EH–domain containing 2	MGEPS
Rn.17695	NM_001107037	Epx	Eosinophil peroxidase	–
Rn.74906	NM_001172809	Ercc2	Excision repair cross–complementing rodent repair deficiency, complementation group 2	–
Rn.19370	NM_001107296	Ercc6	Excision repair cross–complementing rodent repair deficiency, complementation group 6	–
Rn.10798	NM_012557	Fancc	Fanconi anemia, complementation group C	Facc
Rn.3928	NM_144737	Fmo2	Flavin containing monooxygenase 2	–
Rn.54447	NM_012848	Fth1	Ferritin, heavy polypeptide 1	Fth
Rn.8365	NM_012815	Gclc	Glutamate–cysteine ligase, catalytic subunit	Glclc, MGC93096
Rn.2460	NM_017305	Gclm	Glutamate cysteine ligase, modifier subunit	Glclr
Rn.11323	NM_030826	Gpx1	Glutathione peroxidase 1	GSHPx, GSHPx–1
Rn.3503	NM_183403	Gpx2	Glutathione peroxidase 2	GPX–GI, GSHPx–2, GSHPx–GI
Rn.108074	NM_022525	Gpx3	Glutathione peroxidase 3	GSHPx–3, GSHPx–P, Gpxp
Rn.3647	NM_017165	Gpx4	Glutathione peroxidase 4	Phgpx, gpx–4, snGpx
Rn.218434	NM_001105738	Gpx5	Glutathione peroxidase 5	–
Rn.9852	NM_147165	Gpx6	Glutathione peroxidase 6	OBPII, Ry2d1
Rn.4130	NM_001106673	Gpx7	Glutathione peroxidase 7	–
Rn.19721	NM_053906	Gsr	Glutathione reductase	–
Rn.109452	NM_181371	Gstk1	Glutathione S–transferase kappa 1	GST13–13, GSTkappa
Rn.87063	NM_012577	Gstp1	Glutathione S–transferase pi 1	GST–P, Gst3, Gstp, Gstp2, MGC72668, MGC72669

(continued on next page)

Table 1 (*continued*)

Unigene	Gene bank	Symbol	Description	Gene Name
Rn.107334	NM_013096	Hba–a2	Hemoglobin alpha, adult chain 2	HBAM, Hba–a1, Hba1
Rn.3160	NM_012580	Hmox1	Heme oxygenase (decycling) 1	HEOXG, Heox, Hmox, Ho–1, Ho1, hsp32
Rn.1950	NM_031971	Hspa1a	Heat shock 70kD protein 1A	HSP72, Hsp70–1, Hspa1, Hspa1b
Rn.3561	NM_031510	Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble	–
Rn.171849	NM_053792	Ift172	Intraflagellar transport 172 homolog (Chlamydomonas)	Slb
Rn.31789	NM_001008802	Krt1	Keratin 1	Kb1
Rn.112309	XM_346005	LOC367198	Similar to Serine/threonine–protein kinase ATR (Ataxia telangiectasia and Rad3–related protein)	–
Rn.60583	NM_001105829	Lpo	Lactoperoxidase	–
Rn.40511	NM_021588	Mb	Myoglobin	–
Rn.47782	NM_001107036	Mpo	Myeloperoxidase	–
Rn.38575	NM_053734	Ncf1	Neutrophil cytosolic factor 1	Ncf–1, p47phox
Rn.162331	NM_001100984	Ncf2	Neutrophil cytosolic factor 2	–
Rn.64645	NM_033359	Ngb	Neuroglobin	–
Rn.10400	NM_012611	Nos2	Nitric oxide synthase 2, inducible	Nos2a, iNos
Rn.14744	NM_053524	Nox4	NADPH oxidase 4	–
Rn.162651	NM_001100171	Noxa1	NADPH oxidase activator 1	–
Rn.137764	NM_001106986	Noxo1	NADPH oxidase organizer 1	–
Rn.11234	NM_017000	Nqo1	NAD(P)H dehydrogenase, quinone 1	Dia4, MGC93075
Rn.10669	NM_057120	Nudt1	Nudix (nucleoside diphosphate linked moiety X)–type motif 1	Mth1
Rn.30105	NM_057143	Park7	Parkinson disease (autosomal recessive, early onset) 7	CAP1, DJ–1, Dj1, SP22
Rn.2845	NM_057114	Prdx1	Peroxiredoxin 1	Hbp23, MGC108617
Rn.2511	NM_017169	Prdx2	Peroxiredoxin 2	Tdpx1
Rn.2011	NM_022540	Prdx3	Peroxiredoxin 3	Prx3
Rn.17958	NM_053512	Prdx4	Peroxiredoxin 4	MGC72744
Rn.2944	NM_053610	Prdx5	Peroxiredoxin 5	Aoeb166
Rn.42	NM_053576	Prdx6	Peroxiredoxin 6	–
Rn.3936	NM_012631	Prnp	Prion protein	PrP, Prn
Rn.2	NM_001105727	Psmb5	Proteasome (prosome, macropain) subunit, beta type 5	–
Rn.44404	NM_017043	Ptgs1	Prostaglandin–endoperoxide synthase 1	Cox–3, Cox1, Cox3
Rn.44369	NM_017232	Ptgs2	Prostaglandin–endoperoxide synthase 2	COX–2, Cox2
N/A	NM_001100528	Rag2	Recombination activating gene 2	–
Rn.1023	NM_139192	Scd1	Stearoyl–Coenzyme A desaturase 1	–
Rn.4197	NM_173120	Sels	Selenoprotein S	sg2
Rn.1451	NM_019192	Sepp1	Selenoprotein P, plasma, 1	–
Rn.137930	XM_225268	Serpinb1b	Serine (or cysteine) peptidase inhibitor, clade B, member 1b	RGD1560658

Table 1 (*continued*)

Unigene	Gene bank	Symbol	Description	Gene Name
Rn.162022	NM_138832	Slc38a1	Solute carrier family 38, member 1	Ata1, GlnT, Sat1
Rn.81033	NM_138854	Slc38a5	Solute carrier family 38, member 5	SN2
Rn.6059	NM_017050	Sod1	Superoxide dismutase 1, soluble	CuZnSOD
Rn.10488	NM_017051	Sod2	Superoxide dismutase 2, mitochondrial	–
Rn.10358	NM_012880	Sod3	Superoxide dismutase 3, extracellular	ECSODPT
Rn.107103	NM_181550	Sqstm1	Sequestosome 1	Osi, ZIP, ZIP3
Rn.2835	NM_001047858	Srxn1	Sulfiredoxin 1 homolog (S. cerevisiae)	Ab2–390, Npn3
Rn.91199	NM_019353	Tpo	Thyroid peroxidase	–
Rn.29777	NM_053800	Txn1	Thioredoxin 1	Txn
Rn.2758	NM_001008767	Txnip	Thioredoxin interacting protein	MGC94673, Vdup1
Rn.67581	NM_031614	Txnrd1	Thioredoxin reductase 1	MGC93353, Tr
Rn.6300	NM_022584	Txnrd2	Thioredoxin reductase 2	MGC93435, Tr3, Trxr2, Trxrd2
Rn.13333	NM_019354	Ucp2	Uncoupling protein 2 (mitochondrial, proton carrier)	–
Rn.9902	NM_013167	Ucp3	Uncoupling protein 3 (mitochondrial, proton carrier)	–
Rn.2710	NM_031140	Vim	Vimentin	–
Rn.94978	NM_031144	Actb	Actin, beta	Actx
Rn.1868	NM_012512	B2m	Beta–2 microglobulin	–
Rn.47	NM_012583	Hprt1	Hypoxanthine phosphoribosyltransferase 1	Hgprtase, Hprt, MGC112554
Rn.107896	NM_017025	Ldha	Lactate dehydrogenase A	Ldh1
Rn.973	NM_001007604	Rplp1	Ribosomal protein, large, P1	MGC72935

ideal for an efficient NC redox activity. SEM imaging reported in Fig. 1A confirms poly-dispersion of the sample.

Fig. 1B depicts UV/Vis spectrum of NC dispersed in aqueous solution that highlights the typical absorbance peaks at around 220 nm and 310 nm, ascribable to cerium(III) and cerium(IV) respectively [25,26], thus confirming the coexistence of the fundamental species for nanoceria catalytic activity.

Confocal microscopy confirmed the internalization of cerium oxide nanoparticles by PC12 cells. Confocal images are reported in Fig. 2. After 72 h of incubation with 20 μg/ml of Oregon Green labeled NC, cells internalized nanoparticles to a large extent: the internalization process can in fact be appreciated by the presence, inside the cell cytoplasm, of several highly fluorescent green spots. Cytoskeletal f-actin staining in red and nucleus counterstaining in blue were performed for a better interpretation of the images. No evidence of NC in the cell nuclei can be observed. A 3D rendering and a *Z*-stack through confocal microscopy of PC12 cells internalizing fluorescent NC were also performed and reported in the Supplementary material.

Of all the investigated genes after the 72 h treatment, we noticed important statistically significant differences ($p<0.05$) in the transcription of some of these genes, as described in the following. In 20 μg/ml treated samples, Gpx5, Gpx6, Gpx7, Lpo, Ccl5, Fmo2, Krt1, Sepp1 and Ucp3 were down-regulated, while Ncf1, Sod3, and Cygb were up-regulated (Fig. 3A). Instead, in 50 μg/ml treated samples, a down-regulation of Gpx2, Gpx5, Gpx6, Gpx7, Lpo, Ccl5, Fmo2, Krt1, Nos2, Sepp1, Ucp3, and Ngb, and an up-regulation of Hspa1a, Ncf1, Sod3, and Cygb (Fig. 3B) can be pointed out (Table 2).

4. Discussion

The elaboration of antioxidative strategies addressed to the central nervous system (CNS) has a significant relevance since this tissue results to be particularly vulnerable to oxidative stress from a molecular up to a functional level. CNS is in fact abundant in redox-active metals (iron and copper, able to damage DNA), and in fatty unsaturated lipids that may serve as substrates for lipid peroxidation [27]; whereas at a metabolic level, it displays an elevated consumption of oxygen through the mitochondrial respiratory chain [28].

In recent years, the connection between oxidative stress and the onset of several neurodegenerative pathologies like Alzheimer's disease and Parkinson's disease has been well documented [29,30]. In particular, dopaminergic neurons seem to be prone to oxidative stress due to their specific metabolism. Dopamine itself and its metabolites containing two hydroxyl groups are indeed able to exert cytotoxic effects on dopaminergic neurons and surrounding microglia, through the production of superoxide and dopamine quinones. For a comprehensive review of these phenomena, the reader is referred to [31].

Thanks to its well known ROS scavenging properties (mimicking the action of superoxide dismutase and of catalase, enzymes that protect cells against superoxide radicals and H_2O_2, respectively), NC seems to

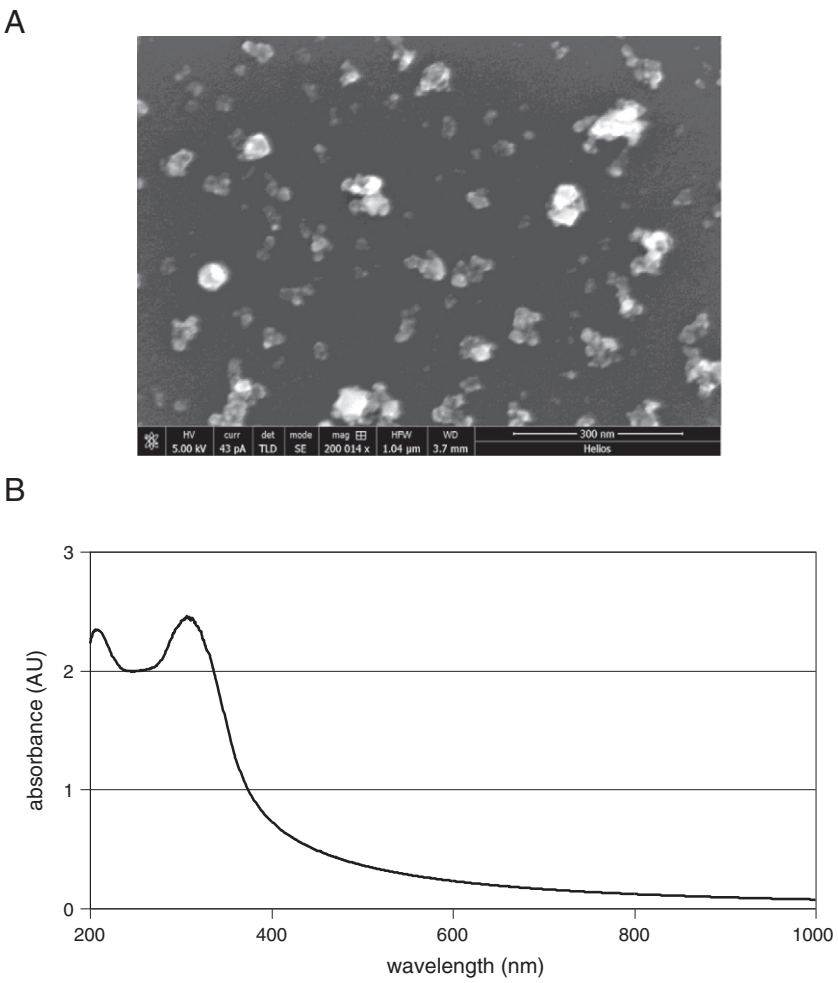

Fig. 1. SEM image (A) and UV/Vis spectrum (B) of cerium oxide nanoparticles in aqueous dispersion.

fulfill well the purpose of reducing oxidative stress in the considered model of neural cells. Highly promising opportunities are thus disclosed for the treatment of different pathologies of the central nervous system. Even in the absence of cellular insults, the alteration of regulation of several genes related to ROS metabolism could indeed provide a good evidence of how NC may intervene in attenuating oxidative conditions and contrasting those events that could result into a pathological state. In many cases, down- or up-regulation of the analyzed genes seems to be dose-dependent; however, for some genes (for instance Gpx2, Ncf1, Sod3), this dependence does not occur. Of course, just two tested concentrations are not enough in order to assess any possible modulation of gene transcription by NC treatment, and these dose-dependent effects will deserve further studies, in order to elucidate any possible mechanism of modulation of the investigated processes due to a broader range of NC concentrations.

In order to facilitate the discussion of the obtained results, we grouped the genes with an altered transcription profile into three categories: genes involved in antioxidant defense (Fig. 4A), genes involved in ROS metabolism (Fig. 4B), and genes coding for oxygen transporters (Fig. 4C).

Genes coding some members of the glutathione peroxidase family (Gpx2, Gpx5, Gpx6, and Gpx7) resulted significantly down-regulated (Fig. 4A). The products of these genes are considered as among the most important components of organism antioxidant defense, in particular in the CNS [32], playing a major role in the reduction of hydrogen peroxide and organic hydroperoxides, by using reduced glutathione [33]. A decrement of their expression suggests a compensatory mechanism triggered by the presence of the nanoparticles: since ROS are largely scavenged by NC, a feedback mechanism induces cells a lower expression of the natural ROS scavengers, because their action is supported/replaced by NC.

Lpo is a gene encoding the enzyme lactoperoxidase, a *heme* containing glycoprotein that catalyzes the oxidation of halides and pseudo-halides by hydrogen peroxide, and generates products owning an important antimicrobial activity [34]. This process is strictly correlated to the presence of superoxide radicals, and the inhibitory effect of superoxide dismutase (SOD) is largely documented [35]: it is thus clear as SOD activity of NC can interfere with Lpo, by reducing the presence of superoxide radicals, and thus decreasing its transcription level (Fig. 4A).

Chemokine (C–C motif) ligand 5 (also CCL5) is a protein encoded by the Ccl5 gene, also known as RANTES (Regulated And Normal T cell Expressed and Secreted), and represents a chemokine that normally acts as a mediator factor able to recruit leukocytes under inflammatory conditions: by infiltration of leukocytes into inflamed sites, tissue damage following oxidative stress typically undergoes exacerbation. In the CNS, this chemokine is secreted by glial cells, and therefore many studies are addressed to elucidating its role into neurodegeneration.

Previous studies devoted to the assessment of gene expression in glial cells showed that, following astrocyte stimulation with an inflammatory cytokine (TNFα), Ccl5 transcription was up-regulated by about three times with respect to control cultures [36]. Moreover, in an attempt at elucidating activated glial role in central neuron inflammation and death, other authors showed how interleukin-1β modulation through antioxidants significantly decreases the release of reactive oxygen species and CCL5 by astrocytes [37]. Other evidences showed that high levels of CCL5 have a strongly positive association with autoimmune

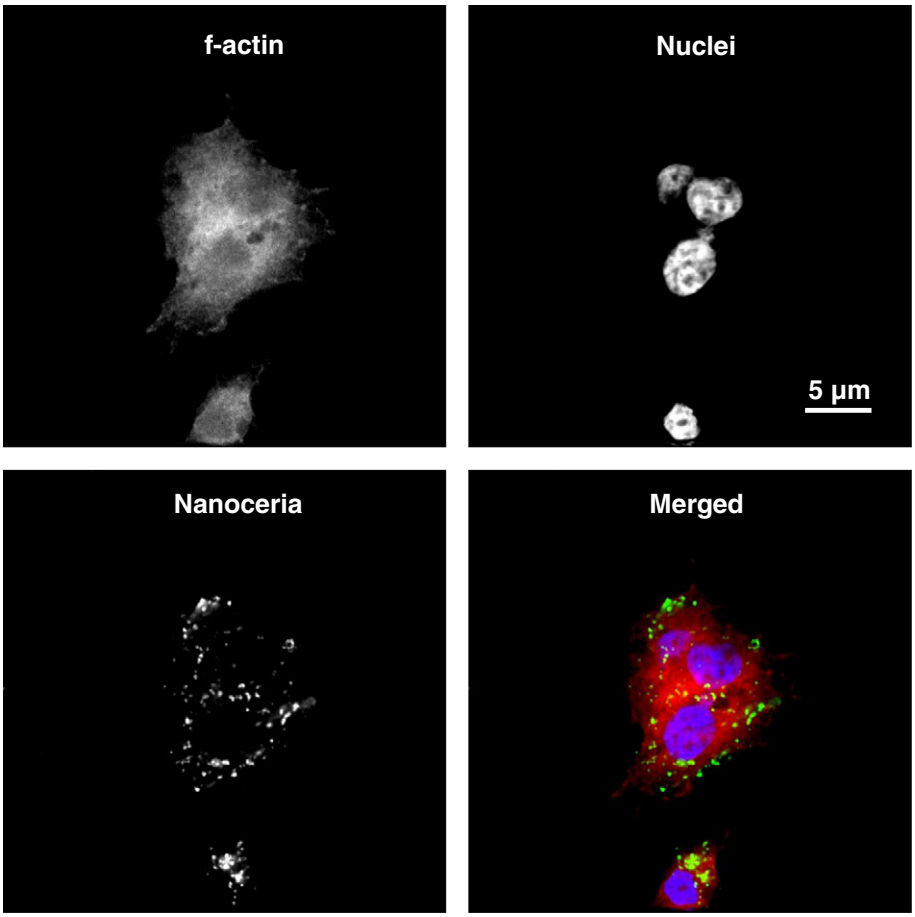

Fig. 2. Confocal images showing nanoceria (in green) internalization by PC12 cells. Cytoskeletal f-actin is stained in red, while nuclei are stained in blue.

diseases like systemic lupus erythematosus [38]. Furthermore, in another anatomical district (*i.e.*, myocardium), CCL5 antagonism was demonstrated as an effective means to increase myocardium recovery in early reperfusion by decreasing leukocyte recruitment after infarction [39].

Considered our finding that Ccl5 transcription is decreased upon administration of NC (Fig. 4B), these studies strongly encourage further investigations on the applicability of cerium oxide nanoparticles as therapeutic agents able to decrease oxidative stress and inflammation that characterize many CNS diseases.

Fmo2 gene encodes flavin monooxygenase 2, an enzyme involved in the oxygenation of several heteroatom-containing nucleophilic compounds, the expression of which undergoes modulation at different levels, with differences related to species, gender and tissue [40]. In recent years, an alteration of Fmo was hypothesized to be correlated with oxidative stress characterizing amyotrophic lateral sclerosis [41]; furthermore, Fmo2 was found to be significantly up-regulated in a mouse model of non-alcoholic steatohepatitis, providing supporting evidence on the relation of oxidative stress and disease state development [42].

Even though the administration of NC was associated to a decreased Fmo2 transcription in our neural cell model (Fig. 4B), being a functional allele coding for full-length transcript of Fmo2 missing in Caucasian humans and occurring at a frequency of 13–20% in African Americans [40], further studies are still required to clarify the significance of this finding.

The intronless gene Hspa1 encodes a 70 kDa heat shock protein (HSP70-1) member of the heat shock protein 70 family, a chaperon protein that enables the cells to face harmful aggregations of proteins during and following an external stress. Its expression therefore confers protection against damages induced by heat, ischemia, and oxidative stress, both *in vitro* and *in vivo* [43]. The over-expression of HSP70 in the nervous system is proven to play a protective role in several different neuronal disease models, protecting neurons from protein aggregation and toxicity, and cells from apoptosis and inflammation [44].

Parkinson's disease, for example, is characterized by the progressive and selective loss of dopaminergic neurons in the *substantia nigra*, and by the presence of intracellular fibrillar α-Syn protein aggregates (Lewy bodies) in the surviving nigral neurons [45]. α-Syn is a 140-amino acid neuronal protein, usually unfolded in aqueous solution, but that can form fibrillar species and insoluble β-sheet-rich fibers [46]. It has been demonstrated that HSP70 is able to reduce α-Syn accumulation, and thus toxicity, in different models of Parkinson's disease [47], by inhibiting α-Syn fibrillar assembly and amyloid formation, and conversely stimulating the formation of amorphous aggregates [48].

A similar neuroprotective role for HSP70 was observed in Alzheimer's disease, a neurodegenerative pathology characterized by a strong extracellular accumulation of amyloid-β peptide (Aβ), jointly to an intracellular accumulation of abnormally phosphorylated *tau* protein as neurofibrillary tangles [49]. HSP70 is able to interact with Aβ structures, inhibiting protein aggregation [50] and incrementing Aβ clearance [51].

Our results, which indicate an up-regulation of Hspa1a in PC12 cells treated with NC (Fig. 4B), thus suggest an indirect protective role of these nanoparticles, and further strengthen their possible application in neurodegenerative pathologies.

Cytokeratins are cytoskeletal filaments with intermediate size, mainly expressed in epithelial cells and in some epithelium-derived tumors, including PC12 cells [52]; among these, keratin 1 (encoded by Krt1

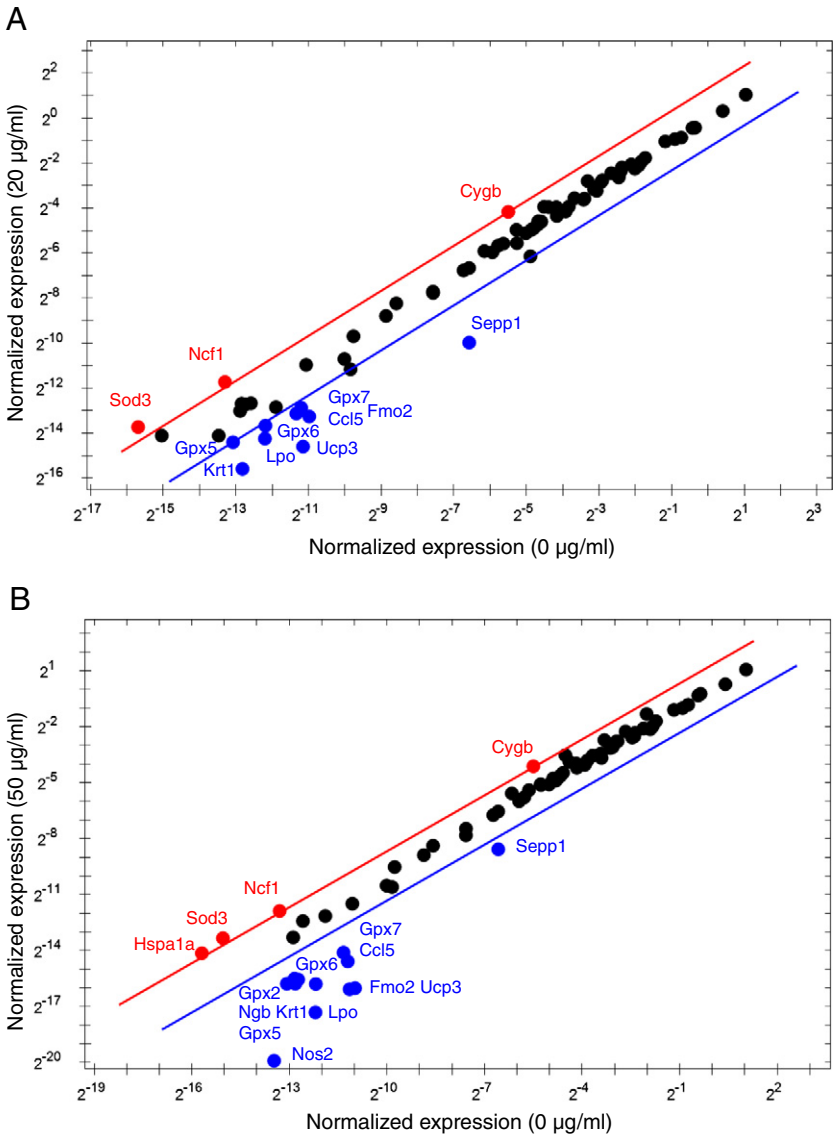

Fig. 3. Scatter plots showing up- and down-regulation of analyzed genes on PC12 cells following treatment with 20 (A) and 50 (B) µg/ml of nanoceria with respect to control cultures (NC = 0 µg/ml).

Table 2
Fold difference (up- or down-regulation) of genes affected by nanoceria treatment.

Gene	Fold difference with respect to 0 µg/ml	
	NC = 20 µg/ml	NC = 50 µg/ml
Ccl5	−3.4	−6.7
Cygb	2.5	2.5
Fmo2	−4.8	−33.3
Gpx2	1.0	−7.1
Gpx5	−2.5	−6.5
Gpx6	−2.8	−12.2
Gpx7	−3.2	−10.4
Hspala	1.9	3.3
Krtl	−6.7	−7.7
Lpo	−4.1	−34.7
Ncfl	3.0	2.7
Ngb	1.1	−6.4
Nos2	−1.6	−86.8
Sepp1	−7.6	−4.0
Sod3	3.9	2.9
Ucp3	−10.9	−30.3

gene) is abundant above the basal membrane where cells are migrating or, in particular, undergoing desquamation [53]. The interest revolving around Krt1 is mainly due to the fact that mutations to Krt1 gene are associated to severe epidermis disorders such as epidermolytic hyperkeratosis [54]. Very few evidences are present in the literature concerning Krt1 expression in different tissues, for example the hepatic one [55], evidencing the correlation between altered Krt1 regulation and oxidative stress. Among these, it is interesting to report that Krt1 mRNA expression was increased upon oxidative stress in human umbilical vein endothelial cells. KRT1 protein expression was significantly increased under the same conditions as well [56]. Our finding of a decreased mRNA expression in PC12 cells upon NC administration (Fig. 4B) may be thus a good indication of a decreased oxidative stress in cells, supporting the need of further studies assessing the significance of Krt1 transcription in the context of central nervous system disorders.

Neutrophil cytosol factor 1 (also known as p47phox) is a protein encoded by the Ncf1 gene, and represents a subunit of neutrophil NADPH oxidase, a multi-component enzyme exploited by cells to produce superoxide anion. It is particularly studied in the field of vascular research, because of its role in vascular oxidative stress and hypertension [57,58]. In the nervous system, p47phox was found to be directly involved in

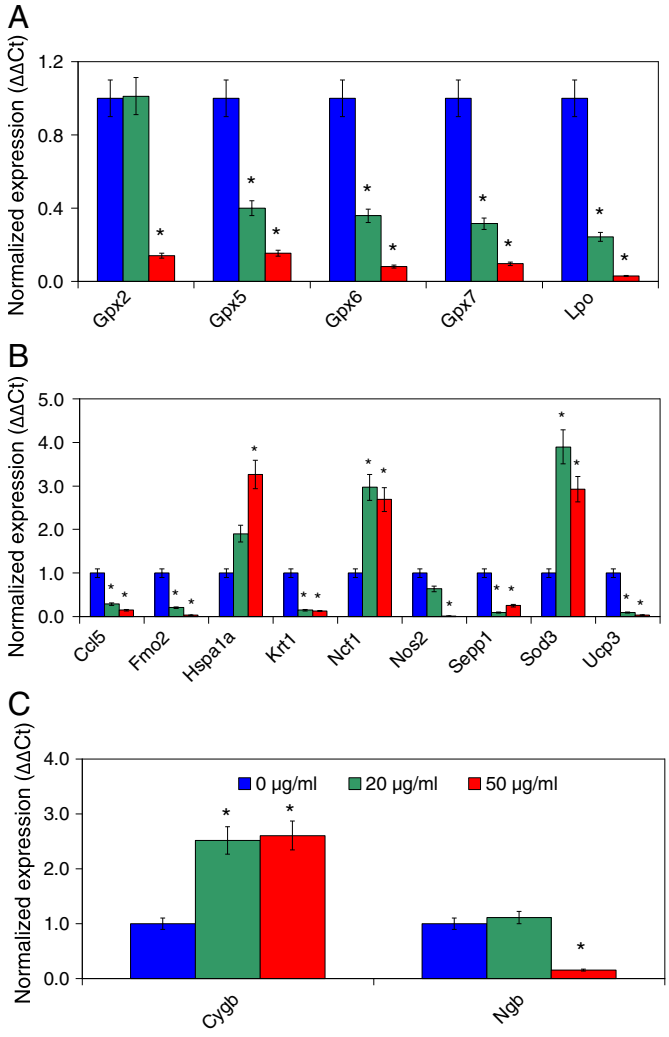

Fig. 4. qRT-PCR analysis of genes with altered transcription: genes involved in antioxidant defense (A), in ROS metabolism (B), and genes coding oxygen transporters (C); $^{*}p < 0.05$.

neuronal oxidative stress and consequent cell death [59], thus an up-regulation of Ncg1 could be seen as a negative effect following administration of NC to the cell culture (Fig. 4B). However, this phenomenon could be explained as a compensatory effect that induces cells to stabilize the levels of ROS following the strong scavenging action of NC, as already seen for Gpx.

Nos2 is a gene that encodes the enzyme nitric oxide synthase (NOS), responsible of the production of the reactive free radical nitric oxide, the up-regulation of which was found in endothelial cells following induction of ROS [60]. In the nervous system, it is directly correlated to inflammatory processes [61], and its over-expression is often followed by neuronal damages and degeneration [62]. Silencing of Nos was found to play a major role in the neuroprotection in models of Parkinson's disease by blocking activation of microglia [63]; thus, a down-regulation of Nos2 following NC administration (Fig. 4B) represents an optimal result in view of its exploitation in the treatment of neurodegenerative diseases. Similar effects were highlighted in a natural antioxidant, quercetin, that was able to exert a neuroprotective effect on PC12 cells through inhibition of the NOS/NO system [64].

Selenoprotein 1 (SEPP1, encoded by Sepp1) is a component of a class of selenoproteins owing strong antioxidant properties demonstrated, for example, in rat prostate cells [65] and in human endothelial cells [66]. In human astrocytes, administered SEPP1 was shown as protective against *tert*-butyl hydroperoxide (TBH)-induced oxidative damage; furthermore, down-regulation of Sepp1 through small interfering RNA negatively affected astrocyte viability, making cells prone to TBH toxic effects, and thus confirming SEPP1 protective role against oxidative stress [67].

Interestingly, SEPP1 expression was found spatially correlated with amyloid-β and neurofibrillary tangles in Alzheimer's disease patients, with a possible involvement of Sepp1 in either disease progression or reaction to clear pathological alterations [68]. Similar spatial correlation was found between SEPP1 and Lewy bodies in Parkinson's disease patients, along with an increased selenoprotein expression with respect to cell density [69].

Although very preliminary, our finding of a decreased transcription of Sepp1 in PC12 cells upon NC administration (Fig. 4B) may be indicative of NC capability of modulating oxidative stress in those processes where Sepp1 is involved, and further studies may be addressed at assessing a possible stabilization of the selenoenzyme intracellular pool as a consequence of NC administration.

Sod3 encodes a member of the superoxide dismutase (SOD) protein family, namely extracellular superoxide dismutase (SOD3), an antioxidant enzyme that catalyzes the dismutation of two superoxide radicals into hydrogen peroxide and oxygen. There are several evidences that confirm as SOD3 can attenuate tissue damage and inflammation [70], and that can be neuroprotective against nitric oxide mediated stress to cerebellar neurons [71,72].

If a Sod3 up-regulation, as noticed in our treatments (Fig. 4B), can be positively considered, a down-regulation of this gene was expected parallel to the compensatory effect observed for the Gpx family genes. The unexpected evidence of Sod3 up-regulation could be explained in view of recent findings about dopamine effects on Sod3 transcription: as we have shown in our recent work, NC causes an increment of dopamine release from PC12 cells [22]. Takano and collaborators demonstrated as dopamine induces an up-regulation of Sod3 (extracellular Sod) in cultured rat cortical astrocytes, but has no effects in the transcription of Sod1 (cytosolic Sod) and Sod2 (mitochondrial Sod) isozyme genes [73]. These results are perfectly in line with our analysis that confirmed the up-regulation of Sod3 following NC treatment, but unaltered transcription of both Sod1 and Sod2.

Ucp3 encodes uncoupling protein 3, a member of a family of mitochondrial anion carrier proteins. Its expression is higher in skeletal muscle and, to a much lower extent, is present also in other tissues, such as brown adipose tissue and heart [74]. Despite the level of homology to Ucp1, the role of which has been established in mitochondrial proton gradient dissipation for non-shivering thermogenesis [75], the role of UCP3 protein is still debated and definitive evidences are still far from being achieved [76]. However, an increasing number of studies seems to point out UCP3 involvement into two different mechanisms: ROS and lipotoxicity mitigation. A detailed discussion of these two mechanisms and their functional implications is reported in a review by Bézaire *et al.* [77], while in the following a few studies concerning Ucp3 gene expression and Ucp3 putative connection to decreased ROS conditions are cited.

In rat gastrocnemius muscle, for example, Ucp3 was found to be strongly under-expressed by capsiate ingestion, implying higher ATP production and consuming processes and/or decreased muscle efficiency [78]. Most relevant to the purposes of the present study is that other members of the same family of genes, namely Ucp4 and Ucp5, were found increasingly expressed in neural cells with increasing MPP^{+}- and dopamine induced toxicity [79,80]. Further studies are however necessary to better clarify the significance of the strong Ucp3 down-regulation upon administration of NC (Fig. 4B), especially in the context of central nervous system disorders.

The last two genes the transcription of which has been altered by the NC treatment are Cygb and Ngb, encoding two proteins involved in oxygen transportation: cytoglobin and neuroglobin, respectively (Fig. 4C), two recently discovered intracellular members of the vertebrate hemoglobin family.

Cytoglobin is located in the cytoplasm of fibroblasts, chondroblasts, osteoblasts, and hepatic stellate cells, where it has been hypothesized to be involved in collagen biosynthesis [81]; in neurons, it has been hypothesized to bind oxygen and to have cell protective properties, besides being involved in ROS (NO)-signaling pathway [82].

Neuroglobin is predominantly expressed in neuronal cells and is supposed to protect them from hypoxic and ischemic insults by acting as a scavenger of ROS [83]: as an example, neuroglobin has been proven to decrease ROS over-production, lipid peroxidation, mitochondrial dysfunction and cell apoptosis/necrosis in PC12 cells treated with amyloid-β [84], and, in general, to act as a ROS scavenger in the same cell model [85].

While it is quite accepted that both cytoglobin and neuroglobin have neuroprotective effects, the mechanisms are still rather unclear. Cytoglobin expression is controlled by the HIF pathway under hypoxic conditions; instead, neuroglobin is probably regulated by the MAPK signal transduction pathway, or by hypoxia-inducible protein binding sites [86]. There are also *in vivo* evidences that point out different physiological roles of these two proteins, and even different sites and patterns of expression [87].

It is therefore clear that, at this point, it is very hard to give a satisfactory explanation for the different trends of transcription of Cygb (up-regulation at 20 and 50 μg/ml) and Ngb (down-regulation at 50 μg/ml) following NC administration, and deeper and more specific investigations are needed, in particular at level of protein expression. However, there are also other evidences, even *in vivo* [88], of contrasting behaviors of these two genes, suggesting different regulation mechanisms.

5. Conclusions

Despite several positive evidences in the literature about NC anti-oxidant efficiency (even *in vivo* [89]), the molecular mechanisms underlying these effects remain largely unexplored, and leave the debate about NC biosafety still open. There are some reports, in fact, claiming NC cytotoxicity [90], that however seems to be strictly related to the biological context and to the physical features of the investigated nanoparticles [91].

In order to gain a better insight at gene level on the consequences of NC administration, we performed a gene transcription investigation on PC12 cells treated with 20 and 50 μg/ml of NC for 72 h.

We highlighted, first of all, a general down-regulation of transcription of genes involved in the anti-oxidant defense of the cells (*i.e.*, for example, Gpx2, Gpx5, Gpx6 and Gpx7). This could suggest a negative effect of NC on PC12 cells. However, in view of the previously demonstrated lack of toxicity, of ROS generation and, on the contrary, thanks to the proven anti-ROS activity of NC in PC12 [22], we suggest that cells deployed compensatory response to NC activity: NC acts as a strong exogenous ROS scavenger, and therefore the natural defense mechanisms are attenuated. Similar behavior was highlighted for other genes involved in ROS metabolism, like Ncf1 and Sepp1. An analogous trend was instead not found for Sod3, most probably because of a higher secretion of dopamine following NC administration [22]. The hypothesis of the role of NC as ROS scavenger, that acts as "substitute" of the natural cell defenses, is supported by the strong down-regulation of Lpo, a gene encoding for an enzyme active in the presence of H_2O_2, and by the down-regulation of Krt1 and Ccl5, the latter coding an important chemokine involved in inflammatory processes.

Finally, indirect neuroprotective effects are suggested by the up-regulation of Hspa1 and by an extremely high down-regulation of Nos2, the silencing of which represents an interesting approach for the treatment of many neurodegenerative diseases.

Of course, this study represents just the starting point for a whole comprehension of NC effects at molecular levels: some results are still unclear (see for example, the opposite trend in transcription of Ngb and Cygb), and, for sure, an investigation at protein level has to be carried out; moreover, effects of nanoparticles characterized by different sizes and production processes have to be assessed. Notwithstanding, in our opinion the collected findings further support the exploitation of NC in future treatments of neurodegenerative disease, even at pre-clinical level [92].

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.10.009.

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