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The neuronal differentiation process involves a series of antioxidant proteins

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Summary. Involvement of individual antioxidant proteins (AOXP) and antioxidants in the differentiation process has been already reported. A systematic search strategy for detecting differentially regulated AOXP in neuronal differentiation, however, has not been published so far. The aim of this study was to provide an analytical tool identifying AOXP and to generate a differentiation-related AOXP expressional pattern.

The undifferentiated N1E-115 neuroblastoma cell line was switched into a neuronal phenotype by DMSO treatment and used for proteomic experiments: We used two-dimensional gel electrophoresis followed by unambiguous mass spectrometrical (MALDI-TOF-TOF) identification of proteins to generate a map of AOXP.

16 AOXP were unambiguously determined in both cell lines; catalase, thioredoxin domain-containing protein 4 and hypothetical glutaredoxin/glutathione S-transferase C terminus-containing protein were detectable in the undifferentiated cells only. Five AOXP were observed in both, undifferentiated and differentiated cells and thioredoxin, thioredoxin-like protein p19, thioredoxin reductase 1, superoxide dismutases (Mn and Cu-Zn), glutathione synthetase, glutathione S-transferase P1 and Mu1 were detected in differentiated cells exclusively.

Herein a differential expressional pattern is presented that reveals so far unpublished antioxidant principles involved in neuronal differentiation by a protein chemical approach, unambiguously identifying AOXP. This finding not only shows concomitant determination of AOXP but also serves as an analytical tool and forms the basis for design of future studies addressing AOXP and differentiation per se.

Keywords: Neuronal differentiation – N1E-115 – Antioxidant proteins – Proteomics – Two-dimensional electrophoresis – Matrix assisted laser desorption/ionization-time of flight mass spectrometry

Introduction

The effect of the redox state on a variety of cellular signalling cascades including control of differentiation has been already demonstrated (Castro-Obregon and Covarrubias, 1996; Kamata et al., 1996; Katoh et al., 1997; Suzukawa et al., 2000; Ni et al., 2001; Hoshino and Igarashi, 2002; Erlejman and Oteiza, 2002; Kohler et al., 2003; Lee et al., 2003). Kamata et al. studied involvement of redox regulation in the nerve growth factor (NGF) signalling pathway and neuronal differentiation (ND) in PC12 cells: N-acetyl-cysteine (NAC) inhibited ND induced by NGF via expression of ras suggesting that ND and NGF signalling are subject to regulation by the cellular redox state (Kamata et al., 1996). Katoh and coworkers showed that hyperoxia and generation of reactive oxygen species (ROS) lead to ND in PC12 cells represented by neurite extension (Katoh et al., 1997). Suzukawa and coworkers reported that NGF transiently increased intracellular ROS and that chemical antioxidants blocked this effect along with differentiation (Suzukawa et al., 2000). The ROS proposed to mediate differentiation seem to be hydrogen peroxide as catalase treatment abolished NGF-induced neurite outgrowth and ROS generation.

These observations formed the basis for the Rationale to systematically investigate into the antioxidant proteins (AOXP) involved, although some antioxidant proteins as superoxide dismutase (SOD), glutathione peroxidase and catalase (CAT) have been already described (Erlejman and Oteiza, 2002).

The aim of our study was to show differential expression of antioxidant proteins in differentiated and nondifferentiated neuroblastoma cells by a method allowing construction of an antioxidant protein pattern. For this

purpose we selected two-dimensional gel electrophoresis with subsequent mass spectrometrical identification of proteins.

This proteomic approach used, warrants concomitant and unambiguous identification of AOXP, independent of antibody availability and specificity (Lubec et al., 2003). Moreover, the use of these techniques enables detection of so far unknown structures or hypothetical proteins, those that were simply predicted from nucleic acid sequences.

And indeed, we identified differentiation-related patterns of antioxidant proteins that confirm and extend previous work and provide the basis for the design of future studies of AOXP in ND.

Materials and methods

Cell culture

N1E-115 (Mouse neuroblastoma cells) cells were obtained from ATCC (CRL 2263) and maintained in DMEM (GIBCO) medium containing 4500 mg/L glucose, L-glutamine, without pyruvate and with 10% fetal bovine serum (FBS), antibiotics penicillin, streptomycin at concentrations 60 μg/ml and 100 μg/ml respectively and incubated in a humidified incubator with 5% CO_2 at 37°C.

Neuronal differentiation

Undifferentiated N1E-115 cells were grown in 150 mm dishes in normal DMEM medium (Gibco) with 10% FBS and ND was induced by a change to DMEM medium that contained 2% FBS and 1.25% dimethylsulfoxide (DMSO). Complete ND was observed after five days in the differentiation medium, cells were harvested by using a cell scraper (Nalge Nunc, Rochester, NY).

Sample preparation

Both, undifferentiated and differentiated N1E-115 cells were washed three times in 10 mL phosphate buffered saline (Gibco BRL), centrifuged for 10 min at 800 g at room temperature and subsequently homogenised with 1.0 ml of sample buffer consisting of 7 M urea (Merck, Germany), 2 M thiourea (Sigma, St. Louis, MO), 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate) (Sigma), 65 mM 1,4-dithioerythritol (Merck), 1 mM EDTA (ethylenediaminetraacetic acid) (Merck), 1 mM PMSF, 0.5% carrier ampholytes and protease inhibitor complete (Roche, Switzerland). After homogenization samples were left at room temperature for 1 h and centrifuged at 150,000 g for 60 min and the supernatant was transferred into Ultrafree-4 centrifugal filter unit (Millipore, Bedford, MA), for desalting and concentrating proteins. Protein content of the supernatant was quantified by Bradford protein assay system (Bradford, 1976). The standard curve was generated using bovine serum albumin and absorbance was measured at 595 nm. All experiments were carried out in triplicate.

Two-dimensional gel electrophoresis (2-DE)

Samples were subjected to 2-DE as described elsewhere (Peyrl et al., 2003a). 0.8 mg protein was applied on immobilised pH 3–10 nonlinear gradient strips in sample cups at their basic and acidic ends. Focusing was started at 200 V and the voltage was gradually increased to 8000 V at 4 V/min and kept constant for a further 3 h (approximately 150000 Vh totally). After the first dimension, strips (13 cm) were equilibrated for 10 min in the buffer containing 6 M urea, 20% glycerol, 2% SDS, 2% DTT and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of DTT. After equilibration, strips were loaded on 9–16% gradient sodium dodecylsulfate polyacrylamide gels for second-dimensional separation. Gels (180 × 200 × 1.5 mm) were run at 40 mA per gel. Immediately after the second dimension run, gels were fixed for 12 h in 50% methanol containing 10% acetic acid and stained with colloidal Coomassie blue (Novex, San Diego, CA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Bio-Rad Laboratories, Hercules, CA), covering the range 10–250 kDa. pI values were used as given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess of dye was washed out from the gels with distilled water and gels were scanned with Imagescanner (Amersham Bioscience, Uppsala, Sweden). Electronic images of the gels were recorded using Photoshop (Adobe) and PowerPoint (Microsoft) software.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)

Spots were excised with a spot picker (PROTEINEER sp^{TM}, Bruker Daltonics, Germany), placed into 96-well microtiter plates and in-gel digestion and MALDI sample preparation was performed by an automated procedure (PROTEINEER dp^{TM}, Bruker Daltonics, Germany). Briefly, spots were excised and washed seven times with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, gel plugs were shrunk by addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. Dried gel pieces were reswollen with 40 ng/μl trypsin (Promega, Madison, WI) in enzyme buffer (consisting of 5 mM Octyl β-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated for 4 hrs at 30°C. Peptide extraction was performed with 10 μl of 1% TFA in 5 mM OGP and directly applied onto a target (AnchorChip^{TM}, Bruker Daltonics, Germany) that was spotted with α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) matrix thinlayer. The mass spectrometer used in this work was an Ultraflex^{TM} TOF/TOF (Bruker Daltonics, Germany) operated in the reflector mode. An accelerating voltage of 25 kV was used. Calibration of the instrument was performed externally with $[M+H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin, and adrenocorticotropic hormones (clip 1–17 and clip 18–39). Each spectrum was produced by accumulating data from 50–200 consecutive laser shots. Spectra were interpreted with the aid of the Mascot Software (Matrix Science Ltd, United Kingdom). For protein search, a mass tolerance of 100 ppm and 1 missing cleavage site were allowed and oxidation of methionine residues was considered. The probability score calculated by the software was used as criteria for correct identification.

Results

Neuronal differentiation

As shown in Fig. 1 (A, B) undifferentiated cells were differentiated into a morphologically neuronal phenotype with neuritic outgrowth and a neuronal network (Clejan et al., 1996). Morphologically differentiated cells were judged by the length of neurites with a length more than 2 times the cell body diameter. These cells were subjected to analysis and revealed $>90\%$ cells with a neuronal phenotype (Soucek et al., 1998; Kubista et al., 2002).

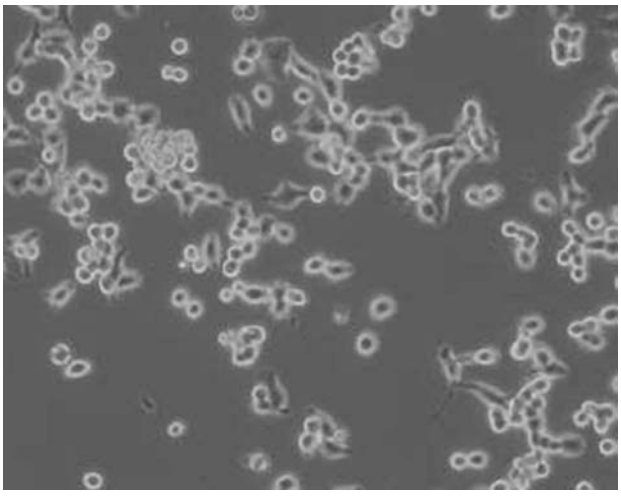

Fig. 1. This figure illustrates the morphological difference between undifferentiated N1E-115 cells (**A**) and differentiated N1E-115 cells presenting with a neuronal network (**B**) (A, B: 10x, objective lens A-plan; Zeiss axiovert 200 microscope)

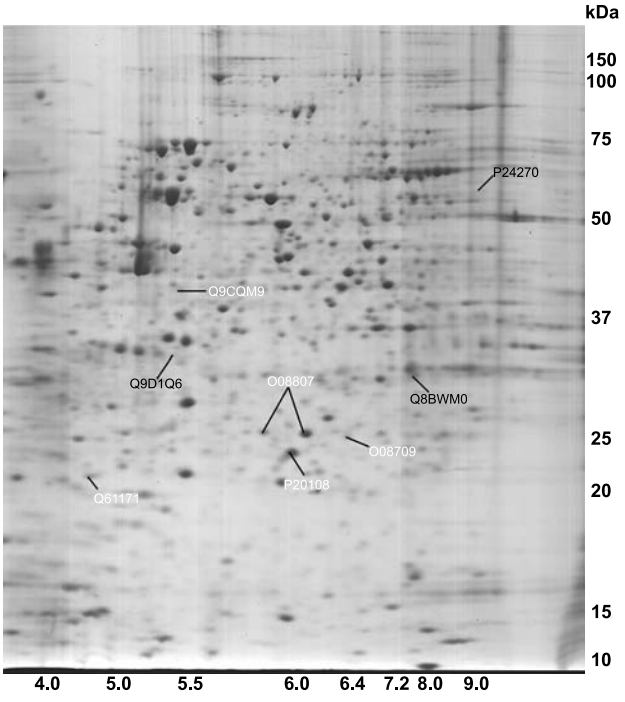

Fig. 2. A master map of the undifferentiated cell line is shown with accession numbers from Swissprot database. 5 out of 8 proteins were observed in both, the undifferentiated and differentiated cell lines (shown with white coloured numbers)

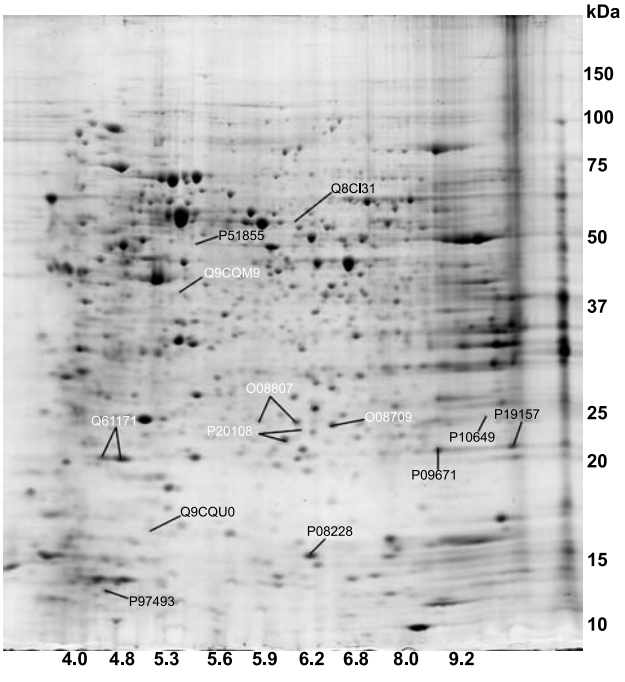

Fig. 3. A master map of the differentiated cell line is presented as above. 13 proteins are shown; 8 proteins were observed in the differentiated cell lines only and 5 proteins (white accession numbers) that were comparable between cell lines are presented above

Protein expression and identification

Both, undifferentiated and differentiated cells were solubilized in the isoelectric focusing reagents containing urea, thiourea and CHAPS and applied on broad-pH-range strips. Second dimensional separation was performed on 9–16% gradient sodium dodecylsulfate polyacrylamide gels and protein spots were visualized by Coomassie blue staining. Experiments were carried out in triplicate. A large series of proteins were expressed in both undifferentiated and differentiated cell lines. Expressed proteins were identified by MALDI-TOF/MALDI-TOF-TOF based upon peptide mass matching.

Table 1. Antioxidant proteins expressed in undifferentiated and differentiated cells

Accession numbers	Name	pI (T)	MW (kDa)	Undifferentiated cells				Differentiated cells			
				Score	Peptide match	pI (O)	Number of spots	Score	Peptide match	pI (O)	Number of spots
P24270	Catalase	7.72	59.63	50	11	9.1	1	–	–	–	–
Q9CQM9	Thioredoxin-like protein 2	5.42	37.78	127	14	5.4	1	108	14	5.35	1
P97493	Thioredoxin, mitochondrial [Precursor]	7.73	18.26	–	–	–	–	116	6	4.5	1
Q9D1Q6	Thioredoxin domain containing protein 4 [Precursor] (Endoplasmic reticulum protein ERp44)	5.09	46.85	83	12	5.35	1	–	–	–	–
Q9CQU0	Thioredoxin-like protein p19 [Precursor] (Endoplasmic reticulum protein ERp19)	5.14	19.05	–	–	–	–	144	11	5.1	1
Q8CI31	Txnrd1 protein (Thioredoxin reductase 1)	5.95	54.34	–	–	–	–	66	13	6.1	1
P20108	Thioredoxin-dependent peroxide reductase, mitochondrial [Precursor]	7.15	28.13	256	13	5.95	1	150	12	6.0 6.1	2
Q61171	Peroxiredoxin 2	5.2	21.78	242	9	4.7 5.85	1	386	14	4.5 4.7 5.85	2
O08807	Peroxiredoxin 4	6.67	31.05	352	14	6.1	2	428	16	6.1	2
O08709	Peroxiredoxin 6	5.72	24.74	159	12	6.35	1	223	22	6.55	1
P08228	Superoxide dismutase [Cu-Zn]	6.03	15.81	–	–	–	–	163	8	6.2	1
P09671	Superoxide dismutase [Mn], mitochondrial [Precursor]	8.8	24.6	–	–	–	–	105	15	8.7	1
P51855	Glutathione synthetase	5.56	52.25	–	–	–	–	90	18	5.45	1
P19157	Glutathione S-transferase P 1	8.13	23.48	–	–	–	–	159	16	9.8	1
P10649	Glutathione S-transferase Mu 1	8.14	25.84	–	–	–	–	84	13	9.5	1
Q8BWM0	Hypothetical Glutaredoxin/ Glutathione S-transferase C terminus containing protein, full insert sequence [Ptges2]	9.1	43.32	73	15	7.5	1	–	–	–	–

Results listing accession numbers, name, theoretical and observed pI, molecular weight, score, peptide matches and number of expression forms (spots). pI (T) means Theoretical pI and pI (O) is Observed pI

From the maps generated (not shown), AOXP were selected and maps were produced for undifferentiated (Fig. 2) and differentiated cell lines (Fig. 3) from three master gels each. Only high-abundance, i.e. Coomassie blue stained spots were used for identification and differences were presented as ''present'' (detectable, i.e. ''switched on'') or ''not-present'' (undetectable, i.e. ''switched off'') thus defining the level of difference. 16 AOXP were identified in both cell lines (Table 1).

AOXPs expressed in undifferentiated & differentiated cells

In the undifferentiated cell line a total of 8 AOXP were identified and most were represented by a single spot except peroxiredoxin 4 that revealed two spots probably reflecting splicing variants or posttranslational modifications (PTM; Fig. 2).

In the differentiated cell line a total of 13 AOXP were identified and thioredoxin-dependent peroxide reductase mitochondrial [precursor], peroxiredoxin 2, peroxiredoxin 4 were represented by two spots each (Fig. 3).

Five AOXP were detected as common to both cell lines and in the undifferentiated cell line three AOXP, catalase, thioredoxin domain containing protein 4 [precursor; syn.:endoplasmic reticulum protein ERp44) and hypothetical glutaredoxin/glutathione S-transferase C terminus containing protein, full insert sequence were identified that were not observed following differentiation (Table 1).

In the differentiated cell line 8 AOXP, mitochondrial thioredoxin, thioredoxin-like protein p19, thioredoxin reductase 1, superoxide dismutases (Cu-Zn and Mn), glutathione synthase, glutathione S- transferase P1 and Mu1 were observed that were not detectable in the undifferentiated cell line (Table 1).

pI shift

Major shifts of pI towards alkaline were observed for CAT, glutathione S-transferase P1, glutathione S-transferase Mu1. Major acidic shifts compatible with phosphorylation or oxidation were observed in thioredoxin, mitochondrial [precursor], thioredoxin-dependent peroxide reductase, mitochondrial [precursor] and hypothetical glutaredoxin/glutathione S-transferase C terminus containing protein, full insert sequence (Table 1, Figs. 2, 3).

Table 2. Domain functions and predicted major localisation of antioxidant proteins

Name	Domain functions	Predicted major localisation
catalase	Catalase is a constitutive peroxisomal enzyme that catalyses the conversion of hydrogen peroxide to water and molecular oxygen in a 'catalatic' reaction, which protects the cell from the toxic effects of hydrogen peroxide. It can also oxidise a number of other compounds in the presence of hydrogen peroxide in a 'peroxidatic' reaction, which is responsible for ethanol oxidation in liver, if the concentration of hydrogen peroxide is high enough. Most catalases exist as tetramers of 65 kDa subunits, each subunit containing a protohaem IX group buried deep within the structure, but which is accessible through hydrophobic channels.	cytoplasmic
thioredoxin-like protein 2	Thioredoxins are small proteins of approximately one hundred amino-acid residues which participate in various redox reactions via the reversible oxidation of an active center disulfide bond. They exist in either a reduced form or an oxidized form where the two cysteine residues are linked in an intramolecular disulfide bond. Thioredoxin is present in prokaryotes and eukaryotes and the sequence around the redox-active disulfide bond is well conserved. Bacteriophage T4 also encodes for a thioredoxin but its primary structure is not homologous to bacterial, plant and vertebrate thioredoxins.	cytoplasmic
thioredoxin, mitochondrial [Precursor]	Thioredoxins are described above.	mitochondrial
thioredoxin domain containing protein 4 [Precursor]	Thioredoxins are described above. This protein also includes endoplasmic reticulum (ER) targeting sequence. Proteins that permanently reside in the lumen of the ER seem to be distinguished from newly synthesized secretory proteins by the presence	cytoplasmic

Table 2 (*continued*)

Name	Domain functions	Predicted major localisation
	of the C-terminal sequence Lys-Asp-Glu-Leu (KDEL). While KDEL is the preferred signal in many species, and is used in vertebrates, Drosophila, Caenorhabditis elegans and plants, variants of that signal are used by different species. The signal is usually very strictly conserved in major ER proteins but some minor ER proteins have divergent sequences (probably because efficient retention of these proteins is not crucial to the cell). Proteins bearing the KDEL-type signal are not simply held in the ER, but are selectively retrieved from a post-ER compartment by a receptor and returned to their normal location. The currently known ER luminal proteins include protein disulphide-isomerase (PDI).	
thioredoxin-like protein p19 [Precursor]	This protein includes a thioredoxin domain and ER targeting sequence described above.	extracellular, including cell wall
txnrd1 protein (thioredoxin reductase 1)	This family includes both class I and class II oxidoreductases. FAD flavoproteins belonging to the family of pyridine nucleotide-disulphide oxidoreductases (glutathione reductase, trypanothione reductase, lipoamide dehydrogenase, mercuric reductase, thioredoxin reductase, alkyl hydroperoxide reductase) share sequence similarity with a number of other flavoprotein oxidoreductases, in particular with ferredoxin-NAD+ reductases involved in oxidative metabolism of a variety of hydrocarbons (rubredoxin reductase, putidaredoxin reductase, terpredoxin reductase, ferredoxin-NAD+ reductase components of benzene 1,2-dioxygenase, toluene 1,2-dioxygenase, chlorobenzene dioxygenase, biphenyl dioxygenase), NADH oxidase and NADH peroxidase. Comparison of the crystal structures of human glutathione reductase and *E. coli* thioredoxin reductase reveals different locations of their active sites.	cytoplasmic
thioredoxin-dependent peroxide reductase, mitochondrial [Precursor]	Peroxiredoxins (Prxs) are a ubiquitous family of antioxidant enzymes that also control cytokine-induced peroxide levels which mediate signal transduction in mammalian cells. Prxs can be regulated by changes to phosphorylation, redox and possibly oligomerization states. Prxs are divided into three classes: typical 2-Cys Prxs; atypical 2-Cys Prxs; and 1-Cys Prxs. All Prxs share the same basic catalytic mechanism, in which an active-site cysteine (the peroxidatic cysteine) is oxidized to a sulphenic acid by the peroxide substrate. The recycling of the sulphenic acid back to a thiol is what distinguishes the three enzyme classes. Using crystal structures, a detailed catalytic cycle has been derived for typical 2-Cys Prxs, including a model for the redox-regulated oligomeric state proposed to control enzyme activity. This family contains proteins related to alkyl hydroperoxide reductase (AhpC) and thiol specific antioxidant (TSA). Alkyl hydroperoxide reductase (AhpC) is responsible for directly reducing organic hyperoxides in its reduced dithiol form. Thiol specific antioxidant (TSA) is a physiologically important antioxidant which constitutes an enzymatic defense against sulphur-containing radicals. This family contains AhpC and TSA, as well as related proteins.	mitochondrial
peroxiredoxin 2	Peroxiredoxins (Prxs) are described above.	cytoplasmic
peroxiredoxin 4	Peroxiredoxins (Prxs) are described above.	cytoplasmic
peroxiredoxin 6	Peroxiredoxins (Prxs) are described above.	cytoplasmic
superoxide dismutase [Cu-Zn]	Superoxide dismutases are ubiquitous metalloproteins that prevent damage by oxygen-mediated free radicals by catalysing the dismutation of superoxide into molecular oxygen and hydrogen peroxide. Superoxide is a normal by-product of aerobic respiration and is produced by a number of reactions, including oxidative phosphorylation and photosynthesis. The dismutase enzymes have a very high catalytic efficiency due to the attraction of superoxide to the ions bound at the active site. There are three forms of superoxide dismutase, depending on	

Table 2 (*continued*)

Name	Domain functions	Predicted major localisation
	the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types. The Fe and Mn forms are similar in their primary, secondary and tertiary structures, but are distinct from the Cu/Zn form. Prokaryotes and protists contain Mn, Fe or both types, while most eukaryotic organisms utilise the Cu/Zn type.	cytoplasmic
superoxide dismutase [Mn], mitochondrial [Precursor]	Superoxide dismutases (SODs) catalyse the conversion of superoxide radicals to molecular oxygen. Their function is to destroy the radicals that are normally produced within cells and are toxic to biological systems. Three evolutionarily distinct families of SODs are known, of which the Mn/Fe-binding family is one. This family includes both single metal-binding SODs and cambialistic SOD, which can bind either Mn or Fe. Fe/MnSODs are ubiquitous enzymes that are responsible for the majority of SOD activity in prokaryotes, fungi, blue-green algae and mitochondria. Fe/MnSODs are found as homodimers or homotetramers. The structure of Fe/MnSODs can be divided into two domains, an alpha N-terminal domain and an alpha/beta C-terminal domain, connected by a loop. The structure of the N-terminal domain consists of a two helices in an antiparallel hairpin, with a left-handed twist. The structure of the C-terminal domain is of the alpha/beta type, and consists of a three-stranded antiparallel beta-sheet in the order 213, along with four helices in the arrangement alpha/beta (2)/alpha/beta/alpha (2).	mitochondrial
glutathione synthetase	Glutathione synthetase (GSS) catalyses the conversion of gamma-L-glutamyl-L-cysteine and glycine to phosphate and glutathione in the presence of ATP. This is the second step in glutathione biosynthesis. In humans, defects in GSS are inherited in an autosomal recessive way and are the cause of severe metabolic acidosis, 5-oxoprolinuria, increased rate of hemolysis and defective function of the central nervous system.	cytoplasmic
glutathione S-transferase P 1	In eukaryotes, glutathione S-transferases (GSTs) participate in thedetoxification of reactive electrophilic compounds by catalysing their conjugation to glutathione. The GST domain is also found in S-crystallins from squid and proteins with no known GST activity, such as eukaryotic elongation factors 1-gamma and the HSP26 family of stress-related proteins, which include auxin-regulated proteins in plants and stringent starvation proteins in *E. coli.* The major lens polypeptide of cephalopods is also a GST. Bacterial GSTs of known function often have a specific, growth-supporting role in biodegradative metabolism: epoxide ring opening and tetrachlorohydroquinone reductive dehalogenation are two examples of the reactions catalysed by these bacterial GSTs. Some regulatory proteins, like the stringent starvation proteins, also belong to the GST family. GST seems to be absent from Archaea in which gamma-glutamylcysteine substitute to glutathione as major thiol. Glutathione S-transferases form homodimers, but in eukaryotes can also form heterodimers of the A1 and A2 or YC1 and YC2 subunits. The homodimeric enzymes display a conserved structural fold. Each monomer is composed of a distinct N-terminal sub-domain, which adopts the thioredoxin fold, and a C-terminal all-helical sub-domain. This entry is the C-terminal domain.	cytoplasmic
glutathione S-transferase Mu 1	GSTs are described above.	cytoplasmic
hypothetical Glutaredoxin/ Glutathione S-transferase C terminus containing protein, full insert sequence [Ptges2]	Glutaredoxin, also known as thioltransferase, is a small protein of approximately one hundred amino-acid residues. It functions as an electron carrier in the glutathione-dependent synthesis of deoxyribonucleotides by the enzyme ribonucleotide reductase. Like thioredoxin, which functions in a similar way, glutaredoxin possesses an active center disulfide bond. It exists in either a reduced or an oxidized form where the two cysteine residues are linked in an intramolecular disulfide bond. Glutaredoxin has been sequenced in a variety of species.	extracellular, including cell wall

Table 2 shows predicted major subcellular localisation and tentative function of the corresponding AOXP from the domain or family (http://www.psort.org, http://www.expasy.org).

Discussion

As shown in the Results, a series of antioxidant proteins were deranged following ND by DMSO-treatment. This differential expressional pattern of AOXP allows insight into molecular mechanisms of the changing redox state during differentiation.

The fact that five AOXP were detected i.e. expressed in both cell lineages, warrants specificity of this process. Differentiation led to disappearance of CAT, thioredoxin domain containing protein 4 and hypothetical glutaredoxin/glutathione S-transferase C terminus containing protein and indeed, CAT has been already reported to be involved in ND: Erlejman and Oteiza revealed decreased CAT protein and activity following dibutyryl cAMP-mediated differentation of human neuroblastoma cell line IMR32 (Erlejman and Oteiza, 2002).

We herein confirm downregulation of CAT to undetectably low CAT protein levels and this suggests a common principle and role of CAT in the ND process, both, in humans and our mouse neuroblastoma cell line. We used DMSO for the induction of ND and as dibutyryl cAMP showed the same effect, we may propose that CAT reduction following ND was due to the ND process per se rather than simply by the two compounds used for the induction of ND.

It remains open, however, if downregulation of CAT and all other decreased protein levels described below were merely due to the decreased proliferation rate observed and inherent to all neuronally differentiated cell lines. Increased proliferation may very well activate oxidative mechanisms and therefore increased AOXP may be increased compensatorily.

On the other hand, addition of CAT to PC12 cells abolished ND and the decreased of CAT observed in our experiment in differentiated N1E-115 cells may be in agreement with findings from Suzukawa and coworkers (Suzukawa et al., 2000).

Thioredoxin domain containing protein 4 (syn.: endoplasmic reticulum protein p44) was no longer detectable on 2D gels following ND. This protein maybe involved in the control of oxidative protein folding (Anelli, 2002). Formation of disulfide bonds is an essential step for maturation and exit of secretory proteins from the ER and p44 maybe a key element of thiol-mediated retention and contribute to quality control and maturation of disulfide-linked oligomeric protein (Anelli et al., 2003). We here add a possible role for p44 in the process of ND.

The hypothetical glutaredoxin/glutathione S-transferase C terminus containing protein, full insert sequence is a protein containing a glutaredoxin, a GST_Cterm and a GST_C_like domain as shown in the InterPro database (http://www.ebi.ac.uk) with a molecular weight of 43323 Da and consisting of 384 amino acids. It has not been described at the protein level so far and is considered a protein predicted from the nucleic acid sequence; this is the first description of its expression in mammalian cells. Based upon the described domains its function is obvious and according to our results this protein may be involved in the mechanism of ND. So far, neither glutaredoxin nor glutathione-S-transferases have been reported to be involved in ND.

In contrast, members of the thioredoxin family as mitochondrial thioredoxin (precursor) and thioredoxin-like protein p19, thioredoxin reductase 1, superoxide dismutases, glutathione synthetase, glutathione S-transferases P1 and Mu1 were upregulated following ND.

Thioredoxin (TRX; mitochondrial) precursor became detectable on the three gels of differentiated neuroblastoma cell line. TRX is a neurotrophic factor and Masutani and coworkers recently demonstrated its relevance for the differentiation process in PC12 pheochromocytoma cells (Masutani et al., 2004). It is upregulated by NGF through the ERK and CREB-CRE signalling cascades and is instrumental for ND. TRX reduces protein disulfides and couples with peroxiredoxins to scavenge ROS. Peroxiredoxins 2, 4 and 6 in the current study were not paralleling the TRX expressional pattern.

The finding of differentiation-related upregulation of TRX and other antioxidants does not fit the tentative explanation from above that AOXP downregulation is mediated by the decrease of the cellular proliferation rate. The involvement of the thioredoxin system in ND of mouse embryonic stem cells (Guo et al., 2001) has been shown by induction of TRX peroxidase 2 following treatment with retinoic acid. This provides evidence that changes of the TRX system are not simply due to chemical agents but to the ND process per se.

ND was also accompanied by an increase of thioredoxin-like protein 19 (precursor; syn.: Erp19), containing a TRX domain with a molecular weight of 19046 Da and consisting of 170 amino acids. This endoplasmic protein was detected in ER preparations and was never described in context with differentiation before. Apart from contain-

ing a TRX domain it serves as protein disulfide isomerase *in vivo* (Knoblach et al., 2003).

Thioredoxin reductase 1 was upregulated following ND in our test system and indeed, thioredoxin reductase is known to regulate AP-1 activity as well as nuclear localisation of TRX via active cysteins (Karimpour et al., 2002); AP-1 in turn is linking ND with the cellular redox state (Leppa et al., 2001). We herein confirm a tentative role of this protein for ND.

The glutathione synthetase was upregulated, i.e. became detectable on 2DE along with two glutathione transferases following ND. Glutathione synthetase, a key enzyme for glutathione biosynthesis, glutathione S transferase P1 and glutathione S-transferase Mu1 were never described to be involved in ND mechanisms and we here propose a role in the redox systems linked to ND.

Superoxide dismutases (SOD) Mn and Cu-Zn were both studied in ND; from ontogenetic studies on olfactory and vomeronasal receptor neurons of rats we learn that SODs expressional patterns are linked to the ND process (Kulkarni-Narla et al., 2003). Erlejman and Oteiza studied susceptibility towards oxidative stress of differentiated vs undifferentiated neurons (Erlejman and Oteiza, 2002). They detected that CuZn superoxide dismutase activity and protein and Mn superoxide dismutase activity were significantly increased in dibutyryl cAMP differentiated human neuroblastoma cells and we now can confirm that increased SODs in our assay system are linked to ND.

Suzukawa and coworkers evaluated the involvement of ROS in NGF-induced ND of PC12 cells (Suzukawa et al., 2000); NGF stimulated in a dose-dependent way generation of ROS and induced ND. CAT was scavenging hydrogen peroxide and prevented ND in terms of significant inhibition of NGF-induced neuritic outgrowth. There is no information whether superoxide species are contributing to the mechanisms of ND and induce SOD expression; we here provide evidence for a tentative role of SODs in relevant antioxidant cascades and redox balance in neuroblastoma cell differentiation. SODs upregulation observed in our study, however, may be seen as a byphenomenon or an innocent bystander and as in the other AOXPs reported herein, functional studies are to follow.

Methodologically, our proteomic approach unambiguously identifies AOXP in N1E-115 cells. Peroxiredoxins, SODs, glutathione handling enzymes etc have been already identified by our group and others in tissues and cells using about one milligram of protein for 2DE and subsequent mass spectrometric identification of proteins (Fountoulakis et al., 2001, 2002; Peyrl et al., 2003a, b; Oh et al., 2004; Shin et al., 2004; Yang et al., 2004). Lower protein levels applied do not show expression of the majority of AOXP (data not shown) using Coomassie staining that in turn represents high-abundance proteins and we here present only manifold up-or downregulation of AOXP, reflecting the outcome of a robust method.

We here demonstrate an AOXP expressional pattern of ND vs undifferentiated cells and show involvement of a series of AOXP in this process that was not reported before. This mapping of AOXP is useful when studies on ND are to be designed and a useful tool for the concomitant and comparative determination of AOXP is provided. Our results are now challenging functional studies on the individual antioxidant structures *in vitro* and *in vivo*.

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