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Protective effect of augmenter of liver regeneration on hydrogen peroxide-induced apoptosis in SH-SY5Y human neuroblastoma cells

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Abstract

Background: Hydrogen peroxide, as other reactive oxygen species (ROS) produced during redox processes, induces lipid membrane peroxidation and protein degeneration causing cell apoptosis. ROS are recently considered as messengers in cell signalling processes, which, through reversible protein disulphide bridges formation, activate regulatory factors of cell proliferation and apoptosis. Disulphide bridges formation is catalysed by sulphydryl oxidase enzymes.

Aim: The neuroprotective effect of ALR protein (Alrp), a sulphydryl oxidase enzyme, on H_2O_2-induced apoptosis in SH-SY5Y cells has been evaluated.

Methods: Cell viability, flow cytometric evaluation of apoptotic cells, fluorescent changes of nuclear morphology, immunocytochemistry Alrp detection, Western blot evaluation of mitochondrial cyt c release and mitochondrial swelling were determined.

Results: Alrp prevents the H_2O_2-induced cell viability loss, apoptotic cell death and mitochondrial swelling in SH-SY5Y cells in culture.

Conclusions: The data demonstrate that Alrp improves SH-SY5Y cells survival in H_2O_2-induced apoptosis. It is speculated that this effect could be related to the Alrp enzymatic activity.

Keywords: *Oxidative stress, neuronal apoptosis, Alrp*

Abbreviations: *ALR, augmenter of liver regeneration; Alrp, augmenter of liver regeneration protein; MTT, 3-[4,5-dimethyl-2-thiazollyl]-2,5-diphenyl-2-tetrazolium bromide; FBS, foetal bovine serum*

Introduction

Oxidative stress, one of the major causes of cellular damage, is a process involved in a variety of human diseases. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical (OH), are the most common oxygen species responsible for cytotoxic effects [1,2]. In particular, H_2O_2, one of the main ROS generated during the redox processes, has been recently considered as a messenger in intracellular signalling [3] and, when it's present or exogenously applied to nucleated eukaryotic cells, it could cause lipid peroxidation and DNA damage which induce apoptosis [4–6].

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Oxidative stress-induced apoptosis is found in many human diseases and, in particular, it represents one of the major causes for cell damage in acute pathological states of the brain, such as ischemia, stroke and trauma [1,7]. In addition it is implicated in the neuropathogenesis of a number of degenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease [8–15].

Unfortunately, the molecular mechanisms involved in oxidative stress-induced neuronal apoptosis are complex and only partially known [16,17]. Recently, attention has been focused, using suitable experimental models, on the identification of molecules with a specific neuroprotective potential against oxidative stress-induced damage. SH-SY5Y cells, a dopaminergic neuroblastoma cell line of human origin, maintained in culture and treated with H_2O_2, represents one of the easiest and more reliable experimental models, generally indicated for evaluation of the protective role of any molecule against hydrogen peroxide-induced apoptosis [6,18,19].

The present study was therefore designed to evaluate the ability of Alrp to prevent oxidative stress-induced cell apoptosis by H_2O_2 on SH-SY5Y cells. Alrp is a liver growth factor [20], which contributes to liver regeneration of partially hepatectomized rats and which is characterized by a strong anti-apoptotic activity [21], probably due to its sulphydryl oxidase enzymatic activity [22].

Materials and methods

Culture cells

SH-SY5Y cells were grown, as previously reported [23], in a complete medium made by DMEM (Sigma-Aldrich, Milan, Italy) supplemented with 10% inactivated FBS (PAA Laboratories GmbH, Austria) and 100 U/ml penicillin/streptomycin and incubated at 37°C in a humid atmosphere of 5% CO_2. The cells were seeded in flasks or Petri dishes or 96-well plates, as required by the different experimental protocols. Cell treatment with complete medium, DMEM supplemented with 10% FBS (control) or supplemented with the specific factors, as planned in each experiment, lasted for 24 h, if not otherwise specified. Alrp recombinant 15 kDa form, obtained as previously reported [24–26], was used. This molecule corresponds to the physiologically active protein that was originally isolated, as reported by Francavilla et al. [27] and it's the one used in most of the experiments, reported in the literature, in which its biological activity has been demonstrated. This issue has recently been discussed by Gao et al. [28].

H_2O_2-induced oxidative stress

To generate oxidative stress on SH-SY5Y cells in culture, H_2O_2, freshly prepared from 30% stock solution prior to each experiment, was used. To determine the most appropriate experimental conditions, we first evaluated cell viability using three different concentrations of H_2O_2 (50, 100 and 200 μM) in the culture medium. On the basis of these results (data not reported) and of the data present in the literature [6], a 100 μM concentration of H_2O_2 was utilized in the subsequent experiments; as control we always referred to cells maintained in complete medium (DMEM supplemented with 10% FBS).

Cell viability

Cell viability was determined using a conventional MTT reduction assay [19]. Briefly, the cells were seeded in a 96-well plate and, after 24 h exposure to the different medium (DMEM + 10% FBS; DMEM + 10% FBS + 100 μM H_2O_2; DMEM + 10% FBS + 100 μM H_2O_2 + 1 ng/ml of Alrp; DMEM + 10% FBS + 100 μM H_2O_2 + 10 ng/ml of Alrp), 20 μl of a solution of MTT (5 mg/ml; Sigma-Aldrich, Milan, Italy) dissolved in Phosphate Buffered Saline-PBS (Sigma-Aldrich, Milan, Italy) were added to each well and each plate was incubated at 37°C for 4 h. The supernatants were then aspirated and 200 μl of dimethyl sulphoxide (DMSO; Sigma-Aldrich, Milan, Italy) were added to each well. The absorbance at 570 nm was measured using a Biorad microplate reader, model 680 (Biorad Laboratories, Milan, Italy), with DMSO as the blank. We chose Alrp concentrations of 1 and 10 ng/ml following the indications of previous experiments. Indeed in earlier experiments we used Alrp at concentrations of 0.2, 0.5 and 1 ng/ml and we detected a good dose-related viability of the cells which was maximal (80%) with 1 ng/ml of Alrp (data not reported). Successively, trying to improve cell viability, we used Alrp at a concentration of 10 ng/ml. With this dosage we obtained a recovery of cell viability improved by more then 10% compared to the 1 ng/ml datum. In light of these results, we decided to use Alrp at a concentration of 10 ng/ml culture medium in all subsequent experiments. All experiments were performed in triplicate.

Immunocytochemistry detection of Alrp

To evidence the Alrp presence in SH-SY5Y cells in basal conditions (D-MEM + 10% FBS) and when Alrp was added to the culture medium (D-MEM + 10% of FBS + 10 ng/ml of Alrp), the cells were grown, for 24 h, in the two experimental conditions, onto coverglasses. After fixing in PFA 4% for 15 min, rinsing with PBS, the cells were permeabilized with 0,25% Triton X-100 for 20 min. After three washes with PBS, non-specific protein binding was blocked by treating the sections with a solution of Foetal Bovine Serum and Bovine Serum Albumin (Sigma-Aldrich, Milan, Italy) in PBS for 2 h. The cells were

then incubated overnight at 4°C with a polyclonal rabbit anti-Alrp antibody (MultiBind GmbH, Koln, Germany) diluted 1:200 in the blocking solution, able to immunodetect Alrp, as already reported in literature [28–30]. The cells were then washed with PBS and incubated with the anti-rabbit ALEXA 488 secondary antibody (Invitrogen SRL, Milano, Italy) diluted 1:200. After washing wit PBS, cells were incubated with TO-PRO-3 (Invitrogen SRL, Milano, Italy) diluted 1:7000 in PBS for 20 min for the nuclear staining. The coverglasses were then mounted with an anti-fading agent (Fluoromount K024, Diagnostic BioSystems, Pleasanton, CA, USA) and analysed with a confocal microscope Leica TCS SP2 (Leica Microsystems, Wetzlar, Germany). To verify the specificity of the immunoreaction, appropriate controls were done incubating the cells with only the secondary Ab or using the pre-immune rabbit serum as primary Ab. We repeated the experiments three times.

Nuclear staining with Hoechst 33342

The cells were grown on coverglasses in a Petri dish, in the presence of the complete culture medium (DMEM + 10% FBS) and exposed, for 24 h, to a solution of 100 μM H_2O_2 alone or in combination with 10 ng/ml Alrp. The coverglasses were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich, Milan, Italy) for 15 min and then washed three times with PBS. Hoechst 33342 (Sigma-Aldrich, Milan, Italy), a DNA fluorochrome (10 μg/ml), was added to the fixed cells and incubated for 20′ at 37°C. After washing in PBS, the coverglasses were mounted and observed by fluorescent microscopy at 480 nm.

Mitochondrial swelling

The cells were grown in 75 cm^2 flasks for 24 h with different medium (DMEM + 10% FBS, control; DMEM + 10% FBS + 100 μM solution of H_2O_2 or DMEM + 10% FBS + 100 μM solution of H_2O_2 and 10 ng/ml of Alrp). Successively the cells were trypsinized (Trypsin 0.05%/ EDTA 0.02%; M-Medical, Milan, Italy), washed with cold PBS and cell pellets were fixed in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/l PBS at pH 7.4 for 5 h at 4°C. Samples were postfixed in 1% OsO4 in PBS for 30 min at 4°C. Fixed samples were washed in several changes of PBS, dehydrated in graded alcohols and embedded in Epon-Araldite (TAAB; Reading, UK). Semithin sections (1 μm thick) were heat-stained with toluidine blue borate [31]. Ultrathin sections for EM were mounted on formvar-coated nickel grids and stained routinely with uranyl acetate and lead citrate [32]. The grids were observed under a Morgagni 268 electron microscope (FEI; Hillsboro, OR).

Flow cytometric detection of apoptotic cells

Annexin V-FITC apoptosis detection kit I (BD Biosciences Pharmingen, San Diego, CA) was used to detect early and late apoptosis and cell necrosis on SH-SY5Y cells incubated in 75 cm^2 flasks with different medium as reported below. Briefly, the cells were exposed, for 24 h, to a culture medium containing DMEM + 10% FBS (control) or DMEM + 10% FBS + 100 μM H_2O_2 or DMEM + 10% FBS + 100 μM H_2O_2 + 10 ng/ml Alrp. Cells were then trypsinized (Trypsin 0.05%/ EDTA 0.02%; M-Medical, Milan, Italy) and washed with cold PBS. One million cells/ml were resuspended in 1 × binding buffer and 5 μl of Annexin V-FITC and 5 μl of PI were added to 100 μl of cell solution and incubated for 15 min at RT in the dark, as suggested by the manufacturer. Four hundred microlitres of 1 × binding buffer were added and cells were analysed by flow cytometry within 1 h.

Determination of mitochondrial cytochrome c release

The cells, incubated in 75 cm^2 flasks for 24 h with different medium (DMEM + 10% FBS, control; DMEM + 10% FBS + 100 μM solution of H_2O_2 or DMEM + 10% FBS + 100 μM solution of H_2O_2 and 10 ng/ml of Alrp), were trypsinized (Trypsin 0.05%/ EDTA 0.02%; M-Medical, Milan, Italy) and washed with cold PBS. For each experimental condition cytosolic proteins, with or without mitochondrial proteins, were extracted following a standard protocol. Protein concentration was evaluated using the Bradford assay (Biorad Laboratories, Milan, Italy). Eighteen micrograms of protein of each sample were loaded in a pre-cast 4–12% SDS-PAGE gel (Invitrogen SRL, Milan, Italy). After a 1-h run, the proteins were transferred onto a PVDF membrane (Biorad Laboratories, Milan, Italy) at 90 mA for 1 h. The membrane was washed with TTBS (Tween-20/Tris-buffered salt solution; Sigma-Aldrich, Milan, Italy) and then incubated in the blocking solution (3% non-fat dry milk in TTBS) for 3 h. After three washes in TTBS, the blocked membrane was incubated overnight at 4°C in the blocking solution containing the primary anti-cytochrome *c* antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation, the membrane was washed three times with TTBS and then incubated in the appropriate HRP-conjugated secondary antibody (Biorad Laboratories, Milan, Italy) for 2 h. After washing with TTBS, the bands were developed using chemiluminescent ECL Plus Western Blotting detection reagents (Amersham Biosciences, GE Healthcare, Milan, Italy). To detect the β-actin housekeeping protein, the membrane was washed in the Restore Western Blot Stripping Buffer (Pierce Biotechnology, Illinois) for 15 min and reprobed for the anti-β-actin antibody diluted 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)

following the same protocol, as for cyt *c* detection. The density of each band revealed was measured with a Biorad microplate reader, model 680 (Biorad Laboratories, Milan, Italy).

Bcl-2 and Bax mRNA quantitative real-time PCR

Bcl-2 and Bax transcriptional levels were assessed by quantitative Real-Time PCR analysis using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Monza, Italy) and human GAPDH as a control.

Cell culture. The cells, incubated in 75 cm^2 flasks for 24 h with different medium (DMEM + 10% FBS, control; DMEM + 10% FBS + 100 μM solution of H_2O_2 or DMEM + 10% FBS + 100 μM solution of H_2O_2 and 10 ng/ml of Alrp), after 24 h in culture, were trypsinized (Trypsin 0.05%/ EDTA 0.02%; M-Medical, Milan, Italy) and washed with cold PBS.

Real-time PCR.

1. *Total RNA extraction, RNA preparation and first-strand cDNA synthesis*: Total RNA was extracted from trypsinized cells using the Rneasy Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using random hexamers and the TaqMan Reverse Transcription Reagents (Applied Biosystems, Monza, Italy) with 3.125 U/μl of MultiScribe Reverse Transcriptase in a final volume of 50 μl.
2. *RT-PCR*: This was performed in 96-well plates on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Monza, Italy). Data collection and analyses were performed using the machine software. Two-step reverse transcription-PCR was performed using first-strand cDNA with a final concentration of 1 × TaqMan gene expression Assay (Bcl-2, Bax and GAPDH) and 1 × TaqMan Universal PCR Master Mix. The final reaction volume was 25 μl. Each sample was analysed in triplicate and all experiments were repeated, at least three times. A non-template control (Rnase-free water) was included on every plate. The thermal cycler conditions were 10 min hold at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. In the first instance, a standard curve and a validation experiment were performed for each primer/probe set. A series of six serial dilutions (20–0.1 ng/μl) of control cDNA were used as a template for each primer/probe set. The assays were supplied as a 20 × mix of PCR primers and TaqMan Minor Groove Binder 6-FAM dye labelled probes with a non-fluorescent quencher at the 3′-end of the probe. Standard curves were generated by plotting the threshold cycle (CT) number values against the log of the amount of input cDNA. CT is the PCR cycle at which an increase in reporter fluorescence above the baseline level is first detected. The average and SD of amount of target gene expressed was normalized to an endogenous reference and is relative to a calibrator and the relative quantization for each sample was plotted in a bar chart using Microsoft Excel software. The endogenous reference used in all of the experiments reported here was human GAPDH. A normal control of cells was used as calibrator in all of the experiments.
3. *Primers and probes*: PCR primers and fluorogenic probes for target gene and endogenous controls were purchased as Pre-Developed TaqMan Assay for GAPDH gene and TaqMan Gene Expression Assay for Bcl-2 and Bax genes (Applied Biosystems, Foster City, CA) with the following sequences: *Bax-F* (5′ GATGCGTCCACCAAG AAGC 3′) and *Bax-R* (5′ CCAGTTGAAGT TGCCGTCAG 3′); *Bcl-2-F* (5′ GATGCG-TCC ACCAAGAAGC 3′), *Bcl-2-R* (5′ CCAGTTGAA GTTGCCGTCAG 3′); GAPDH-F (P/N 4333 764F; Applied Biosystems, Foster City, CA).

Statistical analysis

All results were expressed as mean ± SD and the experiments, in triplicate, were done at least three times. Statistical analysis was performed between the different groups by Student's *t*-test; $P < 0.05$ indicated significant difference.

Results

Effect of Alrp on H_2O_2-induced cytotoxicity

First, we determined the hydrogen peroxide concentration in the culture medium that was able to induce a significant decrease of cell viability, with respect to control cells viability (FBS alone-treatment). As already reported [6], we established that an H_2O_2 concentration between 50–200 μM in the culture medium is sufficient to reduce cell viability by 50–60% (data not reported). For the present study H_2O_2 was used at a concentration of 100 μM, which significantly ($p = 0.0005$) decreases the viability of neuroblastoma cells to 44 ± 11% with respect to the viability of the control group (100%—only FBS treatment). When Alrp was added to the culture medium for 24 h, at a concentration of 1 ng/ml in the presence of 100 μM H_2O_2, it significantly ($p = 0.004$) ameliorates SH-SY5Y cell viability to more than 80%, as compared to the control group (Figure 1). The use of Alrp at a concentration of 10 ng/ml demonstrated a better, even if not statistically significant, effect on cell survival, 10% more compared to the one achieved with a concentration of Alrp of 1 ng/ml. In light of these data, in all subsequent experiments, we used 10 ng/ml of Alrp in the culture

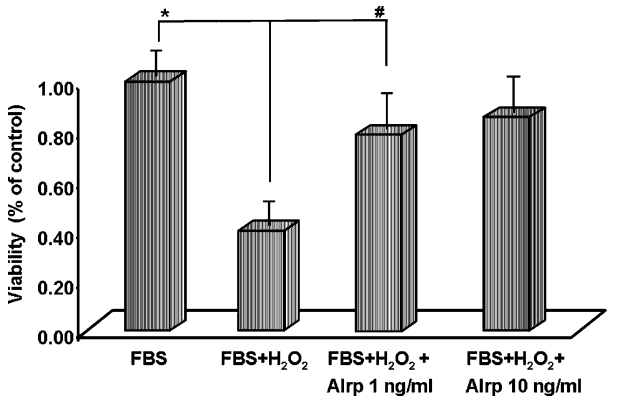

Figure 1. Effect of Alrp on cell viability in H_2O_2-induced cytotoxicity. The cells were exposed to complete medium, DMEM supplemented with 10% inactivated FBS (FBS) or to complete medium supplemented with 100 μM H_2O_2 (FBS+H_2O_2) or to complete medium supplemented 100 μM H_2O_2 and with different concentrations of Alrp (1 and 10 ng/ml medium). After 24 h incubation, cell viability was evaluated by MTT method. Each experiment was done in triplicate. The data are expressed as Mean±SD; $^{\star}p=0.0005$ compared with FBS group; $^{\#}p=0.004$ compared with FBS+H_2O_2 group.

medium. The presence of Alrp alone did not induce any effect on SH-SY5Y cell viability (data not reported).

Immunocytochemistry detection of Alrp

In Figure 2 we report the data obtained evaluating Alrp presence in the cell cytosol when this factor was absent (FBS) or present (FBS+ 10 ng/ml of Alrp) in the culture medium. Figure 2A reports nuclear identification by TO-PRO-3, Figure 2B the specific Alrp immuno-detection and, in Figure 2C, the 'merge' imagine is reported. A low level of immuno-detectable Alrp was documented in the FBS-treated cells, revealing a small quantity of such protein in normal, Alrp untreated, neuroblastoma cells; on the contrary a tremendous intracellular Alrp-related signal was detected when this protein was present in the culture medium, testifying a significant uptake of Alrp by the cells.

Effect of Alrp on H_2O_2-induced release of cytochrome c *from mitochondria into the cytosol of SH-SY5Y cells*

After 24 h of SH-SY5Y cell incubation, as reported in M&M, proteins from whole cell cytosol or from mitochondria-free cytosol were prepared and cyt *c* evaluated by WB analysis. When neuroblastoma cells were incubated in presence of H_2O_2 an increase of cyt *c* in the mitochondria-free cytosol was registered, which, in part, was reduced when Alrp was present in the culture medium (Figure 3A). No differences on cyt *c* levels, determined on whole cell cytosol, were registered among the three experimental conditions. The densitometric analysis of the immunochemical signals of cyt *c* detected in the mitochondria-free cytosol, normalized by the respective *β*-actin values, revealed a statistically significant ($p=0.003$) increase of cyt *c* to ~2.5-fold, compared to the control value, when neuroblastoma cells were incubated in the presence of H_2O_2 alone (Figure 3B). The contemporary presence of Alrp in the culture medium, at the

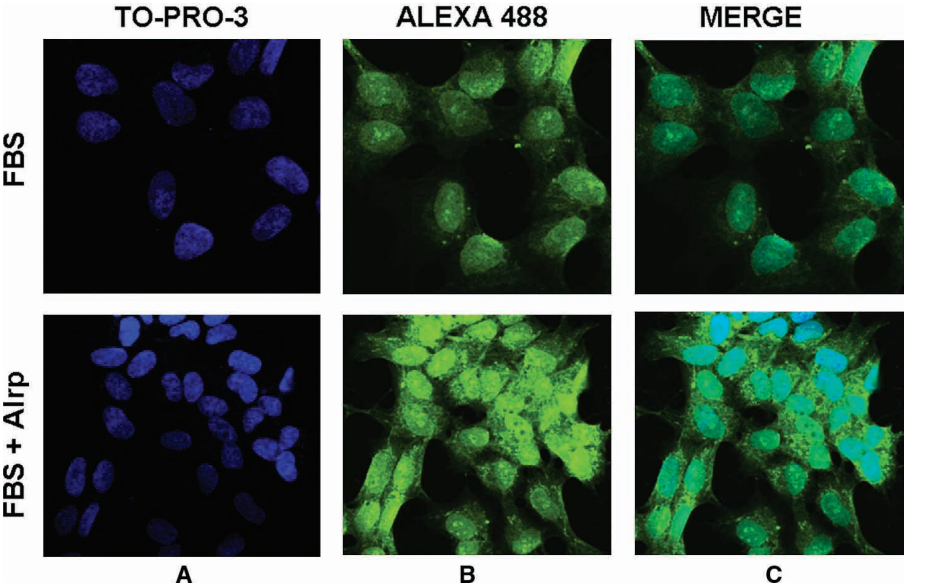

Figure 2. Immunocytochemistry detection of Alrp in SH-5YSY cells. The cells were grown onto coverglasses for 24 h with complete medium (D-MEM+10% of FBS) added or not with Alrp (D-MEM+10% of FBS+10 ng/ml of Alrp). The cells were then incubated overnight at 4°C with a polyclonal rabbit anti-Alrp antibody diluted 1:200 in the blocking solution and, after washing with PBS, incubated with the anti-rabbit ALEXA 488 secondary antibody diluted 1:200. After washing in PBS, cells were incubated with TO-PRO-3 diluted 1:7000 in PBS for 20 min for the nuclear staining. The coverglasses were then mounted with an anti-fading agent and analysed with a confocal microscope Leica TCS SP2. (A) Nuclear identification by TO-PRO-3, (B) the specific Alrp identification. (C) The 'merge' imagine is reported. The experiment was repeated at least three times.

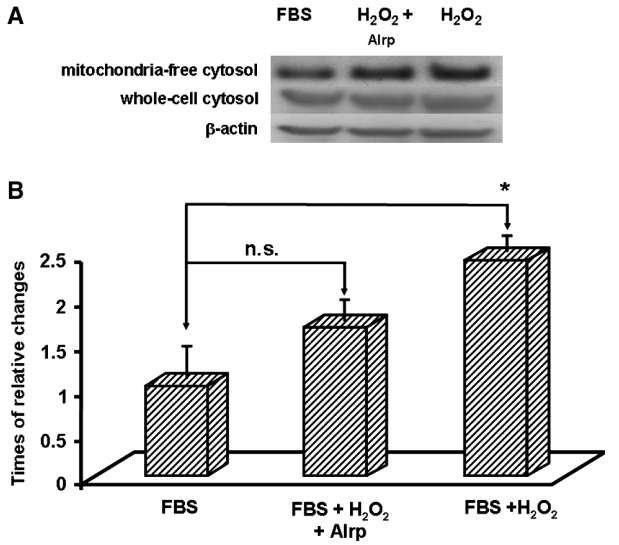

Figure 3. Effect of Alrp on H_2O_2-induced mitochondrial cyt *c* expression changes in mitochondrial-free cytosol. The cells, incubated in 75 cm^2 flasks for 24 h in the presence of complete medium, DMEM supplemented with 10% inactivated FBS (FBS) or of complete medium supplemented with 100 μM H_2O_2 (H_2O_2) or of complete medium supplemented with 100 μM H_2O_2 and 10 ng/ml medium of Alrp, were trypsinized and washed with cold PBS. For each experimental condition cytosolic proteins, with or without mitochondrial proteins, were extracted. Eighteen micrograms of proteins of each sample were loaded in a precast 4–12% SDS-PAGE gel and, after 1-h run, the proteins were transferred onto a PVDF membrane at 90 mA for 1 h. The membrane was then incubated overnight at 4°C in the blocking solution containing the primary anti-cytochrome *c* antibody. After incubation, the membrane was washed three times with TTBS and then incubated with HRP-conjugated secondary antibody and the bands were developed using chemiluminescent ECL plus Western Blotting detection reagents. The density of each band revealed was measured with a Biorad microplate reader, model 680. The densitometric values are expressed as mean ± SD and derive from three different experiments. $*p = 0.003$ compared with the FBS group value.

concentration used in our experimental conditions, partially inhibited, more then 30%, cyt *c* release.

Effect of Alrp on H_2O_2-induced apoptosis of SH-SY5Y cell

The membrane-permeable DNA-binding dye Hoechst 33342 was used to asses the protective effect of Alrp on H_2O_2-induced apoptosis of SH-SY5Y cells. In control cultures, nuclei exhibited uniformly stained chromatin (Figure 4A). However, following 24 h of H_2O_2 treatment, typical apoptotic changes were observed, including an increased number of cells with nuclear condensation and bright staining in morphology under fluorescent microscopy (Figure 4B). These data confirm the capacity of hydrogen peroxide to induce cell death, at least in part, via an apoptotic mechanism. The addition of Alrp (10 ng/ml culture medium) significantly prevented the changes of nuclei morphology induced by H_2O_2 (Figure 4C).

Figure 5 reports the data of flow cytometric analysis of SH-SY5Y cells treated for apoptotic cell identification, as reported in M&M. Analysing neuroblastoma cells, four populations could be identified: *viable cells* in the lower-left quadrant (low PI and FITC signals); *early apoptotic cells* in the lower-right quadrant (low PI and high FITC signals); *necrotic cells* in the upper-left quadrant (high PI and low FITC signals) and *late apoptotic or necrotic cells* in the upper-right quadrant (high PI and high FITC signals). The flow cytometric data of SH-SY5Y cells in the three experimental conditions are reported in the upper section of Figure 5. Control culture cells flow cytometric analysis showed viable cells and a small amount of apoptotic cells, low PI and high FITC signals (A); in contrast H_2O_2-treated cells revealed an increased presence of apoptotic cells (B), which was partially prevented by the presence of Alrp added to the culture medium of H_2O_2-treated cells (C). The statistical evaluation of the data is reported in the lower section of Figure 4. H_2O_2 presence in culture medium induced a statistically significant ($p = 0.0004$) increase of early apoptotic cells, which was partially, but significantly ($p = 0.007$), inhibited by Alrp presence in the culture medium of H_2O_2-treated cells.

It is well known that Bcl-2 and Bax ratio is a marker of induced or inhibited apoptosis (for extensive reviews on apoptosis, see [33,34] and [35]). As recently reported, H_2O_2-treatment of SH-SY5Y cells alters Bcl-2/Bax expressions, up-regulating Bcl-2 and down-regulating Bax, which induces apoptotic cell death [36]. We investigated whether Alrp could have any effect on this process. As shown in Figure 6, our data revealed that the presence of H_2O_2 in the culture medium significantly ($p = 0.005$) down-regulated Bcl-2/Bax mRNA levels; on the contrary, the presence of Alrp in the culture medium of H_2O_2-treated cells significantly ($p = 0.005$) restored Bcl-2/Bax mRNA ratio.

Protective effect of Alrp on mitochondrial swelling

Mitochondrial swelling was evaluated by electron microscopy. SH-SY5Y cells were cultured for 24 h in the presence of DMEM supplemented with 10% FBS (control) or DMEM with 10% FBS added with hydrogen peroxide (100 μM) or DMEM with 10% FBS added with hydrogen peroxide (100 μM) and Alrp (10 ng/ml). FBS-treatment of neuroblastoma cells did not affect mitochondrial ultrastructure (Figure 7; 11 000× magnifications): all the observed organelles appeared regular and mitochondria showed regular shape and structure (Figure 7; 22 000×; arrows). H_2O_2-treatment of SH-SY5Y cells dramatically alters mitochondria structure: more than 90% of the mitochondria appeared swollen, in great measure vacuolated and showing partial destruction of the cristae and voluminous secondary lysosomes

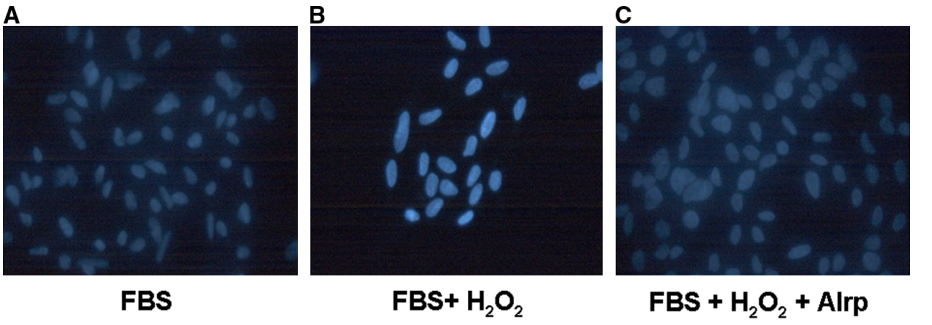

Figure 4. Effect of Alrp on the H_2O_2-induced changes in nuclear morphology. The cells were grown on coverglasses in a Petri dish for 24 h in the presence of the complete culture medium, DMEM+10% FBS (A) or of complete medium supplemented with a solution of 100 μM H_2O_2 alone (B) or in combination with 10 ng/ml Alrp (C). The cells were fixed in 4% paraformaldehyde for 15 min and then washed three times with PBS. Hoechst 33342 (10 μg/ml) was then added to the fixed cells and incubated for 20′ at 37°C. After washing in PBS, the coverglasses were mounted and observed by fluorescent microscopy at 480 nm. The experiment was repeated at least three times.

(Figure 8; 14 000× magnifications, arrows). With a greater magnifications (Figure 8, 22 000× magnifications) a particular of the Golgi apparatus and swollen mitochondria with destroyed cristae are remarked (arrows). The contemporary presence of Alrp and H_2O_2 in the culture medium determined the recovery of the integrity of almost all mitochondria (less than 5% of the observed organelles showed shape and structure typical of swollen mitochondria). Figure 9 documents the ultrastructural aspects of contemporary H_2O_2 and Alrp-treatment of SH-SY5Y cells: mitochondria appear significantly better preserved, (14 000× magnifications), compared to sole H_2O_2 treatment, showing a good conservation of the shape and of the cristae structure at higher magnifications (28 000×).

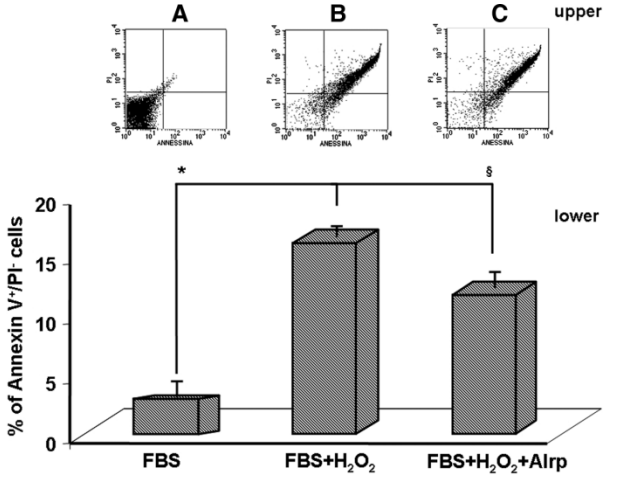

Figure 5. Effect of Alrp on H_2O_2-induced apoptosis by flow cytometry. Four populations was possible to identify: viable cells in the lower-left quadrant (low PI and FITC signals); early apoptotic cells in the lower-right quadrant (low PI and high FITC signals); necrotic cells in the upper-left quadrant (high PI and low FITC signals); and late apoptotic or necrotic cells in the upper-right quadrant (high PI and high FITC signals). In the upper section of the figure the flow cytometric data are reported. Neuroblastoma cells cultured, for 24 h, in the presence of the complete culture medium, DMEM+10% FBS (A) or in the presence of the complete culture medium supplemented with 100 μM H_2O_2 alone (B) or in combination with 10 ng/ml Alrp (C). $^{*}p=0.0004$ compared with FBS-treated group; $^{\S}p=0.007$ compared to H_2O_2-treated group. The experiments were done three times and the values are expressed as Mean±SD.

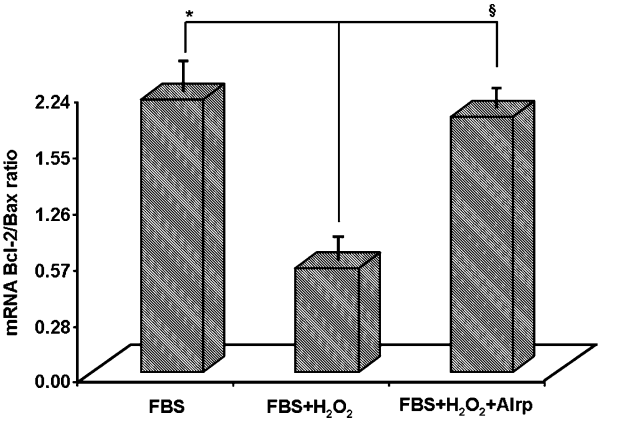

Figure 6. Effect of Alrp on H_2O_2-induced Bcl-2/Bax mRNA down-regulation. Real-time PCR was performed on the ABI Prism 7900HT Sequence Detection System. Two-step reverse transcription-PCR was performed using first-strand cDNA with a final concentration of 1×TaqMan gene expression Assay (Bcl-2, Bax and GAPDH) and 1×TaqMan Universal PCR Master Mix. The final reaction volume was 25 μl. A non-template control (Rnase-free water) was included on every plate. The thermal cycler conditions were 10 min hold at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. In the first instance, a standard curve and a validation experiment were performed for each primer/probe set. A series of six serial dilutions (20–0.1 ng/μl) of control cDNA were used as a template for each primer/probe set. The assays were supplied as a 20× mix of PCR primers and TaqMan Minor Groove Binder 6-FAM dye labelled probes with a non-fluorescent quencher at the 3′-end of the probe. Standard curves were generated by plotting the threshold cycle (CT) number values against the log of the amount of input cDNA. The average and SD of amount of target gene expressed was normalized to an endogenous reference and is relative to a calibrator and the relative quantization for each sample was plotted in a bar chart using Microsoft Excel software. The endogenous reference used in all of the experiments reported here was human GAPDH. A normal control of cells was used as calibrator in all of the experiments. Each sample was analysed in triplicate and all experiments were repeated twice. $^{*}p=0.005$ compared with FBS-treated cells; $^{\S}p=0.005$ compared with H_2O_2-treated cells.

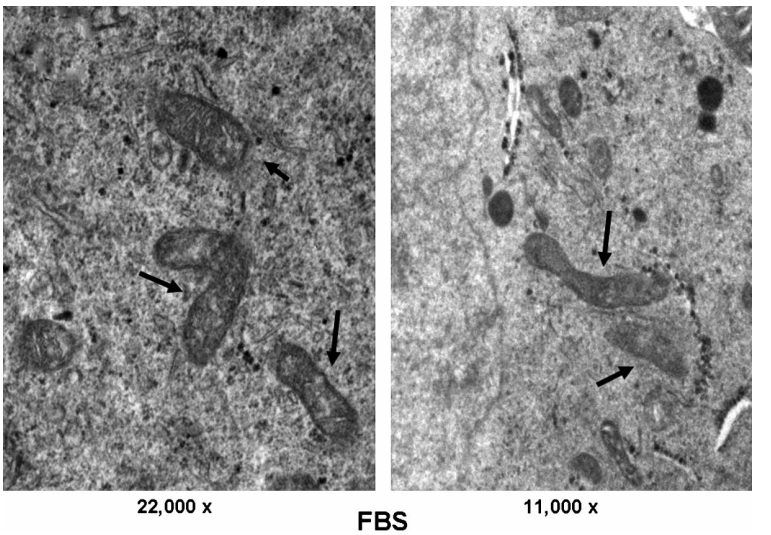

Figure 7. Electron-microscopic analysis of mitochondrial integrity evaluated on FBS-treated neuroblastoma cells. The cells, grown in 75 cm^2 flasks for 24 h in the presence of DMEM+10% FBS, were trypsinized, washed with cold PBS and cell pellets fixed in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/l PBS at pH 7.4 for 5 h at 4°C. Samples were post-fixed in 1% OsO4 in PBS for 30 min at 4°C. Fixed samples were washed in several changes of PBS, dehydrated in graded alcohols and embedded in Epon-Araldite. Semithin sections (1 μm thick) were heat-stained with toluidine blue borate. Ultrathin sections for EM were mounted on formvar-coated nickel grids and stained routinely with uranyl acetate and lead citrate. The grids were observed under a Morgagni 268 electron microscope. The cellular organelles appears regular and undamaged (11 000 × magnifications; arrows) and mitochondria show regular shape and structure (22 000 × magnifications; arrows).

Discussion

In the present paper we report, for the first time, the protective effect of Alrp on H_2O_2-induced apoptosis in SH-SY5Y cells, a dopaminergic neuroblastoma cell line of human origin. This effect has been documented, principally, by (i) the reduction of cell death (Figure 1), (ii) the inhibition of cyt *c* mitochondrial release into the cell cytosol (Figure 3), (iii) the reduction of apoptotic cells (Figures 4 and 5), (iv) the up-regulation on Bcl2/Bax gene transcription

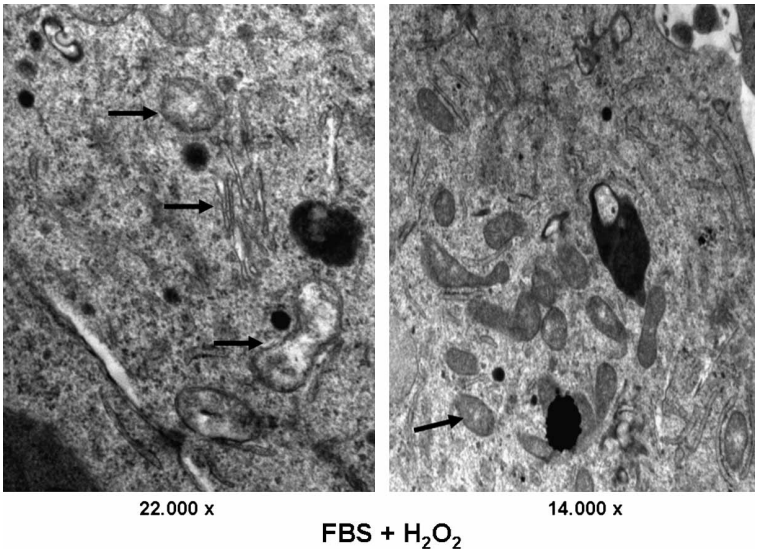

Figure 8. Electron-microscopic analysis of mitochondrial integrity evaluated on H_2O_2-treated neuroblastoma cells. The cells, grown in 75 cm^2 flasks for 24 h in the presence of DMEM+10% FBS+100 μM solution of H_2O_2, were trypsinized, washed with cold PBS and cell pellets fixed in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/l PBS at pH 7.4 for 5 h at 4°C. Samples were post-fixed in 1% OsO4 in PBS for 30 min at 4°C. Fixed samples were washed in several changes of PBS, dehydrated in graded alcohols and embedded in Epon-Araldite. Semithin section (1 μm thick) were heat-stained with toluidine blue borate. Ultrathin sections for EM were mounted on formvar-coated nickel grids and stained routinely with uranyl acetate and lead citrate. The grids were observed under a Morgagni 268 electron microscope. H_2O_2-treatment of SH-SY5Y cells alters mitochondria structure (14 000 × magnifications); swollen mitochondria, in great measure vacuolated and showing partial destruction of the cristae, can be observed (arrows). With a greater magnifications (22 000 × magnifications) a particular of the Golgi apparatus and swollen mitochondria with destroyed cristae are remarked (arrows).

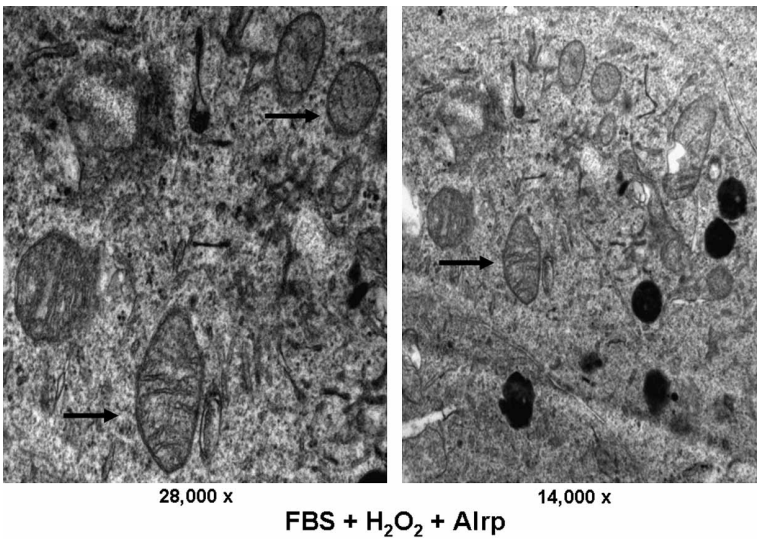

Figure 9. Electron-microscopic analysis of mitochondrial integrity evaluated on H_2O_2- and Alrp-treated neuroblastoma cells. The cells, grown in 75 cm^2 flasks for 24 h in the presence of DMEM + 10% FBS + 100 μM solution of H_2O_2 and 10 ng/ml of Alrp, were trypsinized, washed with cold PBS and cell pellets fixed in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/l PBS at pH 7.4 for 5 h at 4°C. Samples were post-fixed in 1% OsO4 in PBS for 30 min at 4°C. Fixed samples were washed in several changes of PBS, dehydrated in graded alcohols and embedded in Epon-Araldite. Semithin section (1 μm thick) were heat-stained with toluidine blue borate. Ultrathin sections for EM were mounted on formvar-coated nickel grids and stained routinely with uranyl acetate and lead citrate. The grids were observed under a Morgagni 268 electron microscope. The ultrastructural aspects of the cells are reported in the figure: mitochondria appear significantly better preserved (14 000 × magnifications), compared to H_2O_2 alone treatment, showing a good conservation of the shape and of the cristae structure at higher magnifications (28 000 × magnifications).

(Figure 6) and (v) the reduction of mitochondrial swelling (Figures 7–9). All these parameters, altered by H_2O_2 treatment, have been ameliorated by the contemporary presence of H_2O_2 and Alrp in the culture medium.

It is well known that apoptosis is a biological event that is characterized by two different molecular pathways which both lead to activation of caspases: the extrinsic pathway which is triggered by the presence of transmembrane death receptors (Fas, TNF receptor and TRAIL receptors) and their respective ligands on cellular membranes, and the intrinsic pathway, known as mitochondria-mediated apoptosis, which requires the disruption of the mitochondrial outer membrane and the release of mitochondrial inter-membrane proteins, such as cytochrome *c* (Figure 3). Cytochrome *c* release into the cytosol promotes the assembly of apoptosomes, which, in turn, initiate the apoptotic caspase cascade (activation of the initiator of caspase-9, which then cleaves and activates caspase-3). The mitochondrial pathway is suppressed by anti-apoptotic Bcl-2 family proteins (Figure 6), which prevent cytochrome *c* release (for extensive reviews on apoptosis, see [33,34]).

Mitochondrial metabolism is one of the most important cellular activities for all tissues and in particular for the brain. It is, in fact, well known that the physiological activity of the brain mainly depends on mitochondrial-generated energy and on the availability of glucose and oxygen [18]. Oxygen, at the same time, represents a dangerous molecule for the brain when highly reactive species are generated in uncontrolled pathological situations such as an imbalance between pro- and anti-oxidant factors [37]. Indeed, oxidative stress and its damaging agents (ROS) are implicated in many human degenerative disorders of the central nervous system, such as Parkinson's and Alzheimer's diseases [14] and are the main causes of cell loss and mediation of cell apoptosis in Parkinson's and Alzheimer's diseases [8,11,15].

Alrp is a member of a gene family named ALR/ERV1, which includes homologous proteins found in all organisms from viruses to mammals [38]. In eukaryotic cells, these molecules are preferentially localized in the mitochondrial intermembrane space [39]. Many studies demonstrated a tight relation between the biological activity of Alrp and mitochondrial metabolism: (i) Alrp and its yeast homologous ERV1 are proteins essential for mitochondrial functions [40] and directly interact with cytochrome *c* [41], (ii) Alrp and ERV1 are involved in mitochondrial protein import into the intermembrane space and in iron-sulphur cluster assembly in the cytosol [41,42]; (iii) Alrp mRNA, present in all mammalian tissues, is particularly abundant in liver, testis, nerve cells and muscle, all tissues characterized by a metabolism dependent on high mitochondrial activity [43], (iv) Polimeno et al. [30], using normal human muscular tissues, identified the presence of Alrp in the mitochondrial inter-membrane space close to the mitochondrial cristae.

The present data demonstrated that Alrp protects human-derived neuroblastoma cells in culture with

hydrogen peroxide and that this effect could depend on its ability to protect mitochondrial integrity. We already referred to the close relationship between mitochondrial biogenesis and some components of the ALR/Erv1 gene family [30,38–42]. In particular, we have reported an increase of oxygen consumption and ATP production in mitochondria isolated from liver of normal Alrp-treated rats compared to mitochondria liver-derived from normal albumin-treated rats [44].

Recently, Alrp and ERV1 have been considered as sulphydryl oxidase enzymes [22,39]. The ALR/ERV1 protein family is characterized by a well-conserved C-terminal part [38] where a CxxC domain, responsible for sulphydryl oxidase enzymatic activity, is present [22]. Sulphydryl oxidase enzymes catalyse oxidization of thiol residues in cysteines inducing inter- and/or intra-protein disulphide bridges. These enzymes constitute an important component of the biological process known as 'redox regulation', on which depends the reversible oxidation of cysteine residues within key proteins such as enzymes, transcription factors and receptors controlling cell proliferation, apoptosis and cell differentiation.

The protective effect of Alrp on neuroblastoma cells, here reported, leads us to consider Alrp as a factor which may play an important role in neuronal cell metabolism and could contrast the oxidative stress-induced cell damage characteristic of neurological pathologies [1,7–15].

Recently, many researchers have attempted to identify molecules able to attenuate oxidative stress-related neuronal apoptosis. Zhang [6] demonstrated the protective effect of salidroside, a phenylpropanoid glycoside isolated from Rhodiola rosea L, on peroxide-induced apoptosis in SH-SY5Y cells, Chetsawang [18] reports that melatonin protects against peroxide-induced cell death signalling in SH-SY5Y cultured cells.

In conclusion, with the present data we identified Alrp as a physiological molecule able to protect neurological cells from oxidative stress-induced cell death preventing, probably through its sulphydryl oxidase enzymatic activity, mitochondrial membrane swelling and the hydrogen peroxide-related apoptosis.

Further experiments on the molecular substrate of the Alrp enzymatic activity, whose identification will share some light on the biochemical pathway through which Alrp exerts its protective effect, are in progress in our laboratory.

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