Oxidative stress in NPC1 deficient cells: protective effect of allopregnanolone

Stefania Zampieri [a], Synthia H. Mellon [b], Terry D. Butters [c], Marco Nevyjel [a], Douglas F. Covey [d], Bruno Bembi [a, e, *], Andrea Dardis [a]

[a] Unita di Malattie Metaboliche, IRCCS Burlo Garofolo, Trieste, Italy
[b] Department of Obstetrics, Gynecology and Reproductive Sciences, The Center for Reproductive Sciences, University of California, San Francisco, CA, USA
[c] Department of Biochemistry, University of Oxford, Oxford, United Kingdom
[d] Department of Developmental Biology, Washington University in St. Louis, School of Medicine, St. Louis, MO, USA
[e] Regional Coordinator Centre for Rare Diseases, University Hospital "Santa Maria della Misericordia" , Udine, Italy

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Abstract

Niemann-Pick C disease (NPC) is an autosomal recessive neurodegenerative disorder caused by the abnormal function of NPC1 or NPC2 proteins, leading to an accumulation of unesterified cholesterol and glycosphingolipids (GSLs) in the lysosomes. The mechanisms underlying the pathophysiology in NPC disease are not clear. Oxidative damage is implicated in the pathophysiology of different neurological disorders and the effect of GSL accumulation on the intracellular redox state has been documented. Therefore, we determined whether the intracellular redox state might contribute to the NPC disease pathophysiology. Because the treatment of NPC mice with allopregnanolone (ALLO) increases their lifespan and delays the onset of neurological impairment, we analysed the effect of ALLO on the oxidative damage in human NPC fibroblasts. Concentrations of reactive oxygen species (ROS) and lipid peroxidation were higher in fibroblasts from NPC patients than in fibroblasts from normal subjects. Fibroblasts from NPC patients were more susceptible to cell death through apoptosis after an acute oxidative insult. This process is mediated by activation of the NF-κB signalling pathway. Knockdown of NPC1 mRNA both in normal fibroblasts and in human SH-SY5Y neuroblastoma cells caused increased ROS concentrations. ALLO treatment of fibroblasts from NPC patients or NPC1 knockdown cells reduced the levels of ROS and lipid peroxidation and prevented peroxide-induced apoptosis and NF-kB activation. Thus, these findings suggest that oxidative stress might contribute to the NPC disease and ALLO might be beneficial in the treatment of the disease, at least in part, due to its ability to restore the intracellular redox state.

Keywords: Niemann-Pick C • oxidative stress • allopregnanolone

Introduction

Niemann-Pick C disease (NPC) is a lethal, neuronopathic autosomal recessive neurodegenerative disorder of childhood caused by mutations in the NPC1 or NPC2 proteins, leading to an accumulation of unesterified cholesterol and glycosphingolipids (GSLs) in lysosomes [1, 2]. Clinically, NPC is characterized by progressive neurological deterioration and hepato-splenomegaly, with varying age at onset and ensuing course.

Two disease-causing genes, NPC1 (NM000271) and NPC2 (NM006432), have been identified [3, 4]. About 95% of human NPC disease is caused by mutations in the NPC1 gene [5]. Both NPC1 and NPC2 are required for the proper intracellular lipid trafficking. However, the precise role of these proteins is unclear and the molecular mechanisms linking NPC1 and NPC2 mutations to the NPC phenotype are not fully understood.

Oxidative damage has been implicated in the pathophysiology of cancer [6], diabetes [7], atherosclerosis [8], Alzheimer's disease (AD), Parkinson disease (PD) [9], aging [10] and Gaucher disease [11]. Furthermore, treatment of primary cultured cortical neurons with U18666A, a class-2 amphiphile which inhibits cholesterol metabolism and transport from the endosomes/lysosomes to the endoplasmic reticulum [12], leads to apoptosis associated with oxidative stress [13], suggesting a possible role for oxidative damage in the pathophysiology of NPC disease.

*Correspondence to: Dr. Bruno BEMBI,
Coordinator Centre for Rare Diseases,
University Hospital "Santa Maria della Misericordia",
Piazzale Santa Maria della Misericordia 15, 33100, Udine, Italy.
Tel.: +39 0432559914
Fax: +39 04325544617
E-mail: bembi.bruno@aoud.sanita.fvg.it

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Neurons and neuroglia that express steroidogenic enzymes are lost in NPC mice, and the synthesis of allopregnanolone (ALLO) is substantially diminished at birth and decreases further over time [14]. Treatment of NP-C mice with ALLO increases the lifespan, delays the onset of neurological impairment, induces a substantial increase of Purkinje and granular cell survival in the cerebellum and reduces cortical ganglioside accumulation [14]. ALLO treatment in NPC mice is also correlated with its ability to activate the pregnane-X-receptor (PXR)-dependent pathways, suggesting that ALLO may mediate some of its effects through PXR [15]. However, the mechanism by which neurosteroids provide neuroprotection is probably multifunctional.

Therefore, we wanted to address whether the loss of NPC1 function leads to oxidative stress and whether ALLO exerts an effect on the intracellular redox status.

Material and methods

Cells culture and treatments

Human fibroblasts were obtained from skin biopsies from five patients affected with the infantile form of NPC disease (3 males and 2 females, age at onset from 2 to 5 years) and three age-matched controls. All NPC patients presented with the classical biochemical phenotype characterized by massive lysosomal accumulation of cholesterol in cultured fibroblasts. The diagnosis was confirmed by sequencing both NPC1 and NPC2 genes. All patients had mutations in the NPC1 gene. This study was approved by the ethical committee of the IRCCS Burlo Garofolo, and written consent was obtained from all subjects.

Fibroblasts from NPC patients (FNPC) and from normal controls (FNC) were cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum (FCS), and 50 mg/ml penicillin/streptomycin (Gibco, Paisley, UK). Twenty-four hours before performing the experiments, the medium was replaced by serum-free medium supplemented with LDL (50 μg/ml) unless otherwise specified.

Human SH-SY5Y neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS, and 50 mg/ml penicillin/streptomycin (Gibco, Paisley, UK).

NPC1-deficient cells were treated with ALLO (10 to 100 nM) (Steraloids, Newport, RI, USA), the synthetic enantiomer of ALLO, *ent*-ALLO (50 nM) [16, 17] or vehicle, in medium without serum and supplemented with LDL 50 μg/ml for 15 min. to 24 hrs.

Inhibition of NPC1 expression by siRNA

Small interfering RNA (siRNA) duplex derived from the human NPC1 sequence were purchased from Ambion (Pre-designed siRNA, ID8092; targets exon 2 of NPC1). Cultures of fibroblasts and SH-SY5Y cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and 48 hrs later were assayed for cholesterol accumulation by filipin staining, or harvested and assayed for NPC1 protein expression and reactive oxygen species (ROS) levels.

Evaluation of intracellular redox state

Determination of intracellular ROS levels

Intracellular ROS levels were assayed using the fluorescent dye 2'7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Molecular Probes, Eugene, OR, USA) [18] both in cells cultured in the presence or absence of serum. Cultured cells were incubated for 30 min. in media with 5 μM H2DCFDA; then harvested, centrifuged for 3 min. at 280 × *g* and resuspended in 500 μl of phosphate buffer solution (PBS) for FACS analysis on a FACScan device (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of lipid peroxidation

Lipid peroxidation was measured as thiobarbituric reactive substances (TBARS) release into the culture medium. Fibroblasts were incubated for 48 hrs in RPMI 1640 medium without phenol red. Then, the medium was centrifuged for 10 min. at 1000 × *g* and TBARS were measured by mixing the supernatant with thiobarbituric acid and 25% trichloroacetic acid and heating at 100°C for 10 min. Butylated hydroxytoluene (0.5 mM) was included to prevent sporadic lipid peroxidation. TBARS were extracted with 3 ml of butanol and the fluorescence was measured at 515 nm excitation and 555 nm emission. TBARS were expressed as nM malondialdehyde (MDA)/10^6 cells using 1,1,3,3-tetramethoxypropane as MDA standard [19].

Assessment of oxidative stress markers

Glutathione reductase (GR) and catalase (CAT) activity determination

Cells ($5–3\times10^6$) were collected, resuspended in 200 μl of PBS and sonicated for 30 sec. GR and CAT activities were measured using commercially available reagents (Bioxytech Catalase-520 kit and BioxytechGR-340 kits, Oxis Health Products, Foster City, CA, USA).

Determination of total reduced (GSH) and oxidize (GSSG) glutathione

Cells (5×10^6) were harvested and resuspended in 200 μl of metaphosphoric acid (5%). For the GSSG determination, an aliquot was treated with 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), a thiol scavenging reagent, for 1 hr. Total GSH and GSSG were assayed using commercially available reagents (Bioxytech GSH/GSSG-412 kit), according to the manufacturer's instructions.

Free radical scavenging activity (DPPH reduction assay)

To determine the free radical scavenging activity, we used a 2,2-diphenyl-picryl-hydrazyl radical (DPPH) reduction assay. Because of its odd electron, DPPH gives a strong absorption at 516 nm. As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes. Thus, it directly reflects the ability of a compound to scavenge the radical, independently of any enzymatic activity [20]. Briefly, different concentrations of ALLO or vehicle were incubated with 2 ml of DPPH, for 30 min. at 37°C. The decrease of DPPH absorbance was measured at 516 nm. N-acetyl cysteine (NAC) was used as positive control.

Evaluation of apoptosis

Apoptosis in cells treated with different concentrations of H_2O_2 (2.5–10 mM) was evaluated by staining of phosphatidylserine exposed on cell membranes with FITC labelled Annexin V [21], according to the manufacturer's instructions (Sigma, St Louis, MO, USA) and analysed by flowcytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Preparation of protein extracts

Cells (5×10^6) were harvested, resuspended in 50 μl of lysis buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 0.5 mM PMSF, pH 7.9), incubated on ice for 10 min. and centrifuged for 10 min. at $800 \times g$ at 4°C. The supernatant was considered the cytoplasmic fraction. The pellet (nuclei) was washed with buffer A and nuclear proteins were extracted in presence of 50 μl of buffer B (10 mM Hepes, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 0.5 mM PMSF, pH 7.9). Nuclear and cytoplasmatic extracts were then analysed for protein content and stored at –80°C.

Western blot analysis

Protein extracts (10 μg) were resolved on 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). After overnight blocking with 2% BSA in PBS-Tween 0.1% (PBS-T), the membranes were probed with anti-NPC1 polyclonal antibody (Novus Biologicals, Littleton, USA), anti-NF-κB polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-IκB-alpha polyclonal antibody (Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4°C. Anti-rabbit HPR conjugated antibody was used as a secondary antibody. Immunoreactive bands were detected by enhanced chemioluminiscence ECL (Amersham, Biosciences, Milan, Italy). The signals were normalized to those obtained for actin, using a polyclonal anti-actin antibody (Sigma).

Filipin staining

Filipin staining was performed using the method described by Blanchette-Mackie *et al.* [22]. Briefly, the cells were rinsed with PBS and fixed with 3% paraformaldehyde. After washing them with PBS, the cells were incubated with 1.5 mg of glycine/ml PBS for 10 min., stained with filipin (0.05 mg/ml, in PBS 10% FCS) for 2 hrs and examined using a Zeiss fluorescence microscope.

Extraction and oligosaccharide release of gangliosides and neutral glycolipids

Cells (10×10^6) were harvested, washed in PBS and centrifuged at $800 \times g$ for 5 min. The pellet was resuspended in 200 μl of water plus 0.1% Triton-X-100 and frozen and thawed 3 times. Cellular lipids were extracted using the method of Svennerholm *et al.* [23], applied to a pre-conditioned C18 column (Waters Sep-Pak) and eluted with 2 ml of methanol followed by 2 ml chloroform:methanol (1:1 v/v). The eluates were dried under N_2, dissolved in equal volumes of 50 mM sodium acetate buffer (pH 5) containing 1 mg/ml sodium taurodeoxycholate and ceramide glycanase, and incubated for 18 hrs at 37°C.

Anthranilic acid (2-AA) labelling, NP-HPLC separation and quantification of glycolipid-derived oligosaccharides

The oligosaccharides released after treatment with ceramide glycanase were analysed using the method of Neville *et al.* [24]. Oligosaccharides were labelled with 2-AA, purified using Spe-ed Amide columns (Applied Separations, Allentown, PA, USA), separated by normal phase high performance liquid chromatography (NP-HPLC) using a 4.6×250 mm TSK gel-Amide 80 column and detected by fluorescence ($\lambda ex = 360$ nm; $\lambda em = 425$ nm). All chromatography was controlled and data collected and processed using Waters Empower software (Waters, Elstree, Herts, UK). Glucose units were determined, following comparison with a 2-AA labelled glucose oligomer ladder external standard and identified by comparison with glucose unit values of 2-AA labelled oligosaccharides prepared from commercially available glycolipids as described above. The results were normalized by cell number and total protein content.

Statistical analysis

Statistical analysis was performed using Student's t-test analysis. $P < 0.05$ was considered as statistically significant.

Results

Analysis of ROS

We first analysed the intracellular levels of ROS in fibroblasts from normal controls (FNC) and from NPC patients (FNPC) (Fig. 1A). ROS were significantly more abundant in FNPC than in FNC, cultured either in the presence (white bars) or absence (black bars) of serum. It is worth noting that this difference is more evident when cells were cultured in the absence of foetal calf serum, suggesting that FNPC may be more sensitive than FNC to serum starvation. We then treated FNPC with different concentrations of ALLO (10–100 nM) for 24 hrs, showing a dose-dependent reduction in intracellular ROS (Fig. 1B). A similar effect was observed when FNPC were treated for 1 hr with 10 mM of N-acetyl cysteine (NAC), a thiol-containing antioxidant precursor of reduced glutathione.

To determine whether the NPC deficiency caused the oxidative stress, we selectively knocked down the expression of NPC1 in primary cultures of fibroblasts and in human SHSY5Y neuroblastoma cells, using small interference RNA (siRNA). Forty-eight hours after transfection, the expression of the endogenous NPC1 protein was almost completely abolished in SHSY5Y cells, whereas it was reduced to 40% of the control in primary cultured fibroblasts (Fig. 2A and B). The down-regulation of NPC1 protein caused a massive accumulation of intracellular unesterified cholesterol in both cell types, assessed by filipin staining, mimicking

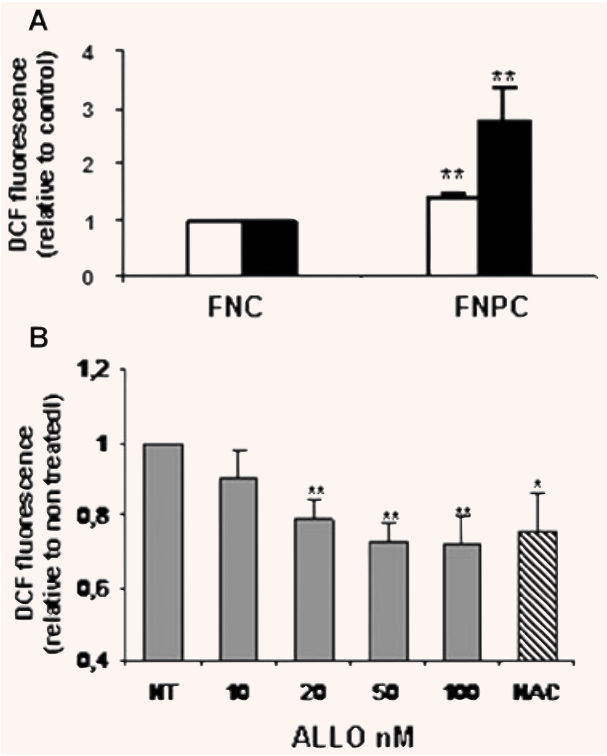

Fig. 1 ROS levels in FNPC are reduced by ALLO.
(**A**) Fibroblasts from NPC patients (FNPC) and normal controls (FNC) were labelled with 5 μM H2DCFDA and analysed by flow cytometry. FNC ($n = 3$) and FNPC ($n = 5$) were cultured in the presence of foetal calf serum (white bars) or in absence of foetal calf serum and 50 ug of LDL (black bars) and dichlorodihydrofluorescein diacetate (DCF) fluorescence was measured. Data (mean ± S.D. of 3 independent experiments) from FNPC are expressed as a percentage of the normal controls. (**B**) DCF fluorescence of fibroblasts from 5 NPC patients treated with the indicated concentrations of ALLO and 10 mM N-acetyl cysteine (NAC) in the absence of foetal calf serum. Data are means ± S.D. of 3 independent experiments. The effect of ALLO or NAC treatment on DCF fluorescence is expressed as percentage of the value obtained from non-treated (NT) cells. ** $P < 0.01$; * $P < 0.05$.

the NPC phenotype (Fig. 2C). The down-regulation of NPC1 protein by siRNA increased ROS significantly in both fibroblasts and neuroblastoma cells, suggesting a causal involvement of NPC1 deficiency in the oxidative stress condition observed in FNPC (Fig. 2D). The association between the down-regulation of NPC1 protein expression and the increase in ROS levels in human SHSY5Y neuroblastoma cells and in normal fibroblasts suggests that NPC1 deficiency would likely lead to a condition of oxidative stress not only in fibroblasts but also in neuronal cells.

Treatment with 50 nM ALLO significantly reduced the intracellular concentration of ROS in NPC knock-down cells to the levels observed in control cells (Fig. 2D). Thus, the protective action of ALLO was readily seen in both fibroblasts and neuroblastoma cell culture system.

To characterize the effect of ALLO on the intracellular redox state in NPC cells further, we compared intracellular ROS levels in FNPC and in SHSY5Y NPC1 knock-down cells treated with ALLO (50 nM) for different periods of time (15 min. to 24 hrs). As shown in Fig. 3 (A and B), there was a significant reduction of ROS after 15–30 min. of treatment, both in FNPC and in SHSY5Y NPC1 knock-down cells. Treatment for more than 30 min. did not reduce ROS levels further. These results suggest that the effect of ALLO on the intracellular redox state might be exerted, at least in part, through a non-genomic mechanism. Furthermore, treatment of both FNPC and SHSY5Y NPC1 knock-down cells with *ent*-ALLO, a synthetic enantiomer of ALLO that interacts with membranes in a manner identical to that of natural ALLO but fails to modulate $GABA_A$ receptor function [16], reduces the intracellular levels of ROS to the same extent as treatment with natural ALLO (Fig. 3C and D).

We determined whether ALLO reduces the intracellular levels of ROS by acting as a free radical scavenger. We assayed, *in vitro,* its ability to scavenge the stable free radical DPPH. As a control, we used the known free radical scavenger N-acetyl cysteine, NAC. As shown in Fig. 4, NAC caused a dose-dependent reduction in DPPH absorbance at 516 nm. However, no differences in DPPH absorbance were observed in the presence of ALLO, suggesting that the ability of ALLO to reduce the intracellular levels of ROS would not be due to a free radical scavenger mechanism.

GR, CAT and GSH/GSSG

To further characterize the intracellular redox state in FNPC, we evaluated the activity of GR and CAT, and the levels of total reduced (GSH) and oxidized (GSSG) glutathione in FNPC and FNC. Although no differences were found in the activity of GR and in the levels of total GSH and GSSG (data not shown), CAT activity was significantly reduced in FNPC with respect to FNC (Fig. 5A). ALLO treatment (50 nM for 24 hrs) partially restored CAT activity to the levels found in FNC (Fig. 5A).

Analysis of lipid peroxidation

To determine whether intracellular accumulation of ROS leads to oxidative damage in FNPC, we measured thiobarbituric reactive substances (TBARS), a marker of lipid peroxidation. TBARS were higher in FNPC than in FNC. ALLO treatment (50 nM, 24 hrs) significantly reduced concentrations of TBARS in FNPC, suggesting that ALLO reduced lipid peroxidation and oxidative damage (Fig. 5B).

Cellular response to oxidative stress

To determine whether impaired intracellular redox state would render FNPC cells more sensitive to apoptosis when exposed to an

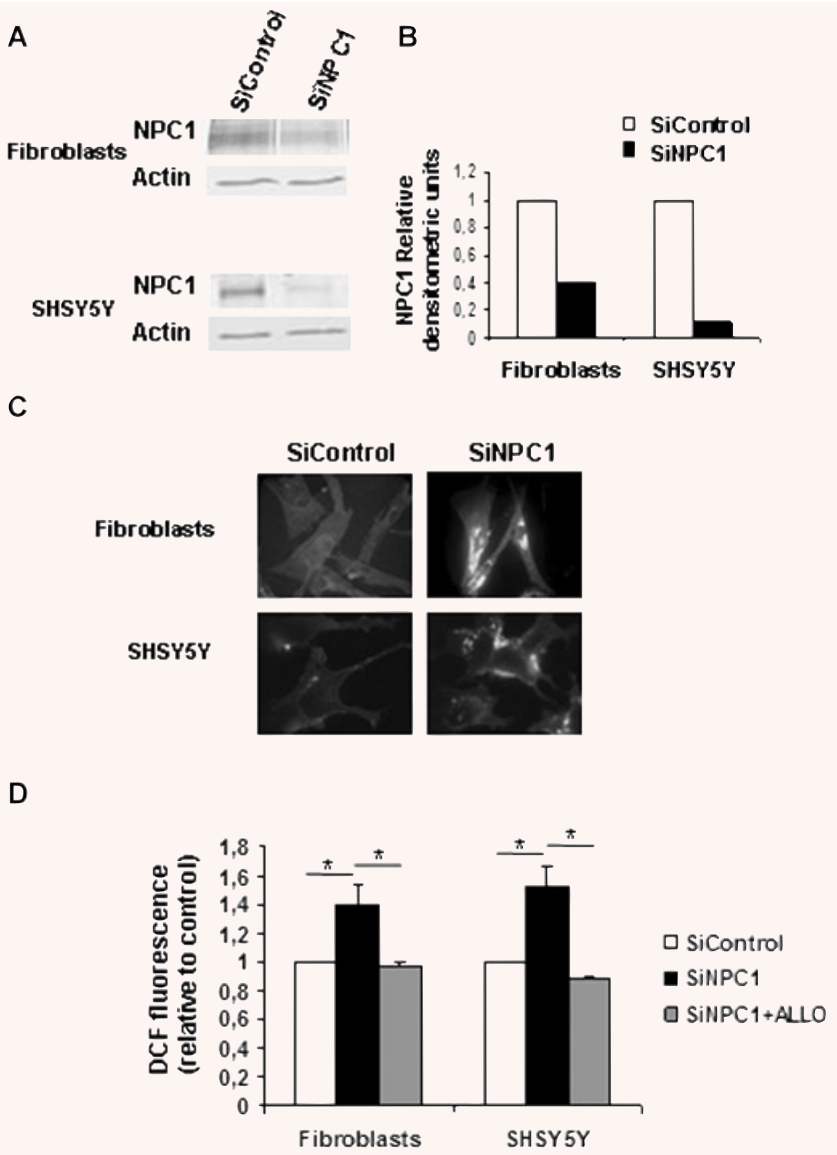

Fig. 2 NPC1 deficiency increases intracellular ROS.
NPC1 protein expression was knocked down by siRNA in primary cultured fibroblasts and in SHSY5Y human neuroblastoma cells. (**A**) Western blot of endogenous NPC1 protein 48 hrs after transfection. (**B**) The intensity of NPC1 signals were normalized against actin and expressed as a percentage of the concentrations found in cells transfected with the control SiRNA (**C**) Filipin staining for cholesterol accumulation revealed massive accumulation of unesterified cholesterol in both cell types. (**D**) Intracellular levels of ROS in NPC1-knockdown cells. ROS was assessed by measuring fluorescence of dichlorodihydrofluorescein diacetate (DCF). Data are mean ± S.D. of 3 independent experiments and are expressed as percentage of the value obtained in cells transfected with siControl ($^*P < 0.05$).

acute oxidative stress, we treated FNPC and FNC with different concentrations of H_2O_2 for 1 hr (Fig. 6) and measured the annexin V immunofluorescence, a marker of apoptosis. Both FNC (white bars) and FNPC (black bars) showed a dose-dependent increase of apoptosis. However, the levels of peroxide-induced apoptosis were significantly higher in FNPC than in FNC (at 2.5 and 5.0 mM of H_2O_2). These data suggest that FNPC are more susceptible to apoptosis after an acute oxidative insult than are FNC. Pre-treatment of FNPC with 50 nM of ALLO for 24 hr significantly reduced the H_2O_2-induced apoptosis, suggesting that ALLO exerts a protective effect against peroxide induced apoptosis (Fig. 6, grey bars). A similar protective effect was observed when FNPC were treated for 1 hr with 10 N-acetyl cysteine, NAC (hatched bars).

NF-κB is a target of ROS in FNPC

A major target of ROS is the transcription factor NF-κB, which exists in the cytoplasm as a dimer bound to an inhibitory protein, IκB. Oxidative stress triggers phosphorylation and degradation of IkB, causing the dissociation of the IκB from the NF-κB, which translocates to the nucleus and activates a variety of target genes [25, 26]. To determine whether peroxide-induced apoptosis in FNPC is associated with increased activation of the NF-κB signalling pathway, we measured the nuclear levels of NF-κB and the cytosolic levels of IκB in FNPC and FNC using Western blot analysis. No differences were found in the basal levels of NF-κB in the nucleus and IκB in the cytoplasm between FNPC and FNC (data

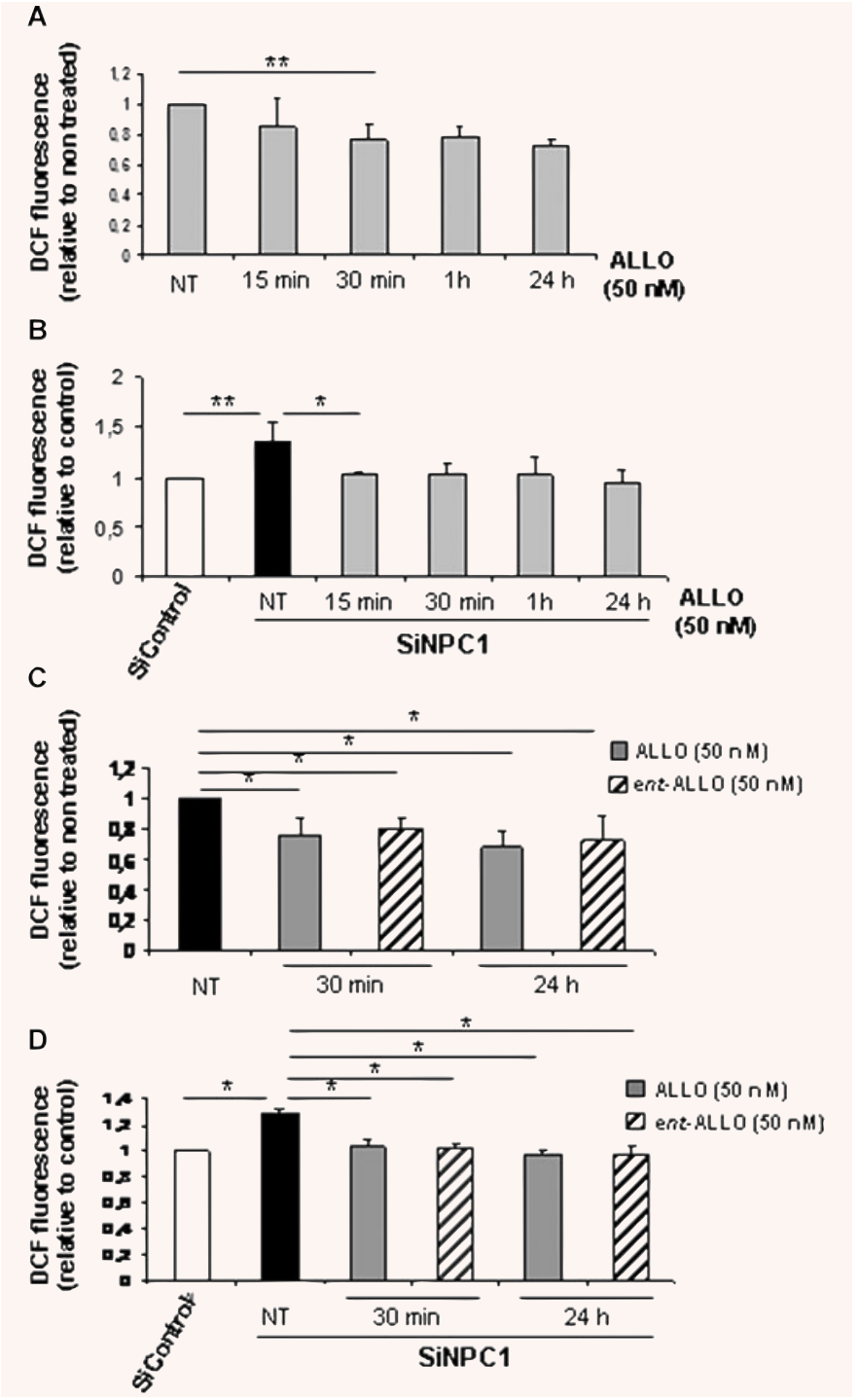

Fig. 3 ALLO might act through a non-genomic mechanisms.
Reactive oxygen species were assessed by measuring fluorescence of dichlorodihydrofluorescein diacetate (DCF). (**A**) DCF fluorescence of FNPC ($n = 5$) treated with 50 nM of ALLO for 15 min. to 24 hrs. The effect of ALLO is expressed as percentage of the value obtained from non-treated (NT) cells (**$P < 0.01$). Data are means ± S.D. of 3 experiments. (**B**) DCF fluorescence in SHSY5Y NPC1-knockdown cells treated with 50 nM ALLO for 15 min. to 24 hrs. Data are means ± S.D. of 3 independent experiments and are expressed as percentage of the value obtained in cells transfected with siControl (**$P < 0.01$, *$P < 0.05$). (**C**) DCF fluorescence of FNPC ($n = 5$) treated with 50 nM of ALLO (grey bars) or *ent*-ALLO (hatched bars) for 30 min. or 24 hrs. The results are expressed as percentage of the value obtained from non-treated (NT) cells (*$P < 0.05$). (**D**) DCF fluorescence in SHSY5Y NPC1-knockdown cells treated with 50 nM ALLO (grey bars) or *ent*-ALLO (hatched bars) for 30 min. or 24 hrs. Data are mean ± S.D. of 3 independent experiments and are expressed as percentage of the value obtained in cells transfected with siControl (*$P < 0.05$).

not shown). However, NF-kB in the nuclei of FNPC increased and IκB in the cytoplasm decreased in response to H_2O_2 treatment, whereas FNC were not responsive (Fig. 7A: Western blots; B and C, quantitation of Western blots).

Under basal conditions, ALLO treatment significantly increased cytosolic IκB in FNPC, whereas nuclear abundance of NF-κB remained unchanged (Fig. 7D, Western blot; E and F, quantitation of blot). When FNPC were pretreated with ALLO before exposure

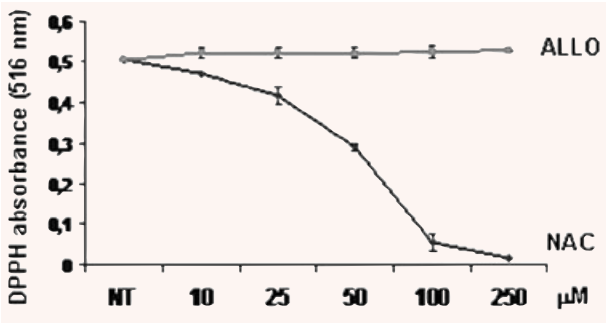

Fig. 4 ALLO does not act as a free radical scavenger.
DPPH spectrophotometric assay based on the quenching effect of radical scavengers on the absorbance of the stable free radical DPPH. ALLO or N-acetyl cysteine (NAC) (10, 20, 50, 100 and 250 uM) were incubated with DPPH (100 uM) for 30 min. at 37°C. Data represent means ± S.D. of 3 experiments each performed in triplicate.

to H_2O_2, nuclear NFkB did not increase and cytosolic IkB did not decrease. Instead, ALLO completely prevented the H_2O_2-stimulated increases in nuclear NF-κB and decreases in cytosolic IκB, suggesting that ALLO might protect NPC cells from increased transcription of NF-κB-dependent genes.

ALLO treatment did not reduce intracellular accumulation of GSLs and cholesterol

We determined whether the effect of ALLO on the intracellular redox state in FNPC was associated to reduced accumulation of GSL or cholesterol. Four FNPC cell lines were treated for 24 hrs with 50 nM of ALLO. GSLs and cholesterol accumulation were analysed by HPLC and filipin staining, respectively. ALLO did not significantly reduce Gb3 and GM3 (the major species detected in fibroblasts, Fig. 8) or cholesterol accumulation (data not shown). Thus, the rapid effects of ALLO on oxidative stress may be independent of long-term changes in GSL or cholesterol accumulation.

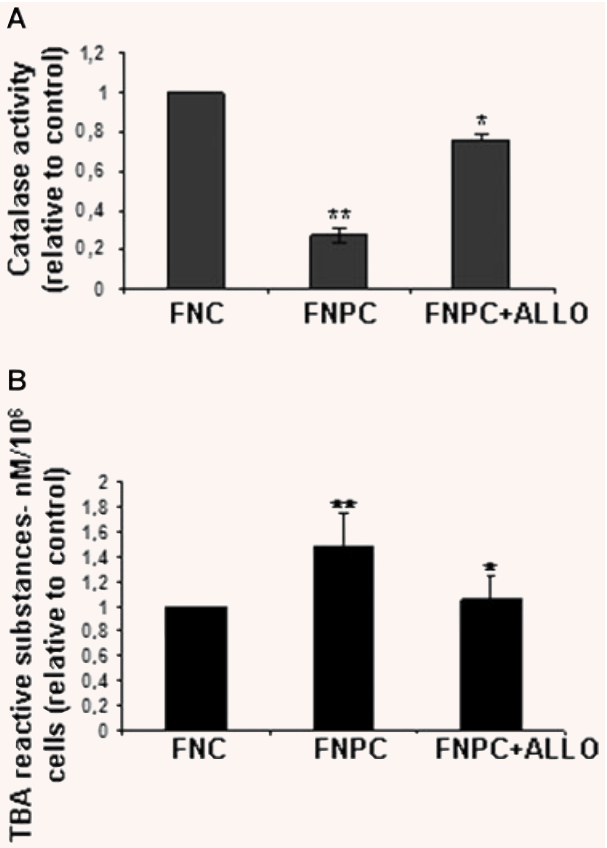

Fig. 5 CAT activity and lipid peroxidation in NPC cells: effect of ALLO treatment
(**A**) Catalase activity was assayed in FNC ($n = 3$) and in FNPC cultured in absence or presence of ALLO 50 nM for 24 hrs ($n = 3$). The activity (units/10^6 cells) is expressed as percentage of the value obtained in FNC. Data are means ± S.D. of 3 independent experiments. (**$P < 0.01$, *$P < 0.05$). (**B**) Lipid peroxidation in FNC ($n = 3$) and in FNPC cultured in the absence or presence of ALLO 50 nM for 24 hrs ($n = 5$) was measured as thiobarbituric reactive substances (TBARs) released into the culture medium. Results are expressed as a percentage of the value obtained in FNC. Data are means ± S.D. of 3 independent experiments (**$P < 0.01$, *$P < 0.05$).

Discussion

Oxidative stress has been implicated in many neurodegenerative disorders [9–11]. Recently, a comparative analysis of gene expression profiles in NPC fibroblasts *versus* normal fibroblasts showed that NPC cells displayed a number of changes in the expression levels of genes involved in the generation of reactive oxygen [27]. Consistent with this observation, we have now shown that fibroblasts from NPC patients have an imbalance in the intracellular redox state that is caused by the deficiency of NPC1, either from a naturally occurring mutation (in patient tissue) or from experimentally induced NPC1 knockdown *in vitro*. Furthermore, NPC1 deficiency leads to an increase in ROS concentration both in fibroblasts and in human SHSY5Y neuroblastoma cells, suggesting that probably the imbalance in the intracellular redox state would not be restricted to a single cell type.

Our data suggest that NPC deficiency leads to decreased CAT activity, which in turn, causes increased intracellular levels of ROS and lipid peroxidation. These findings are in agreement with those found in the NPC mouse model, in which a decrease in CAT activity was found in different tissues. Furthermore, a decrease of several other peroxisomal enzymes has been detected in NPC mouse even before the appearance of clinical symptoms, suggesting that peroxisomal modifications might play a significant role in the ethiology of NPC disease [28].

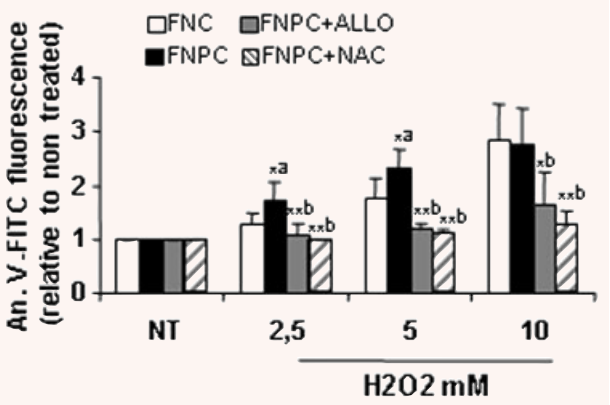

Fig. 6 FNPC are more sensitive to peroxide-induced apoptosis and ALLO treatment exerts a protective effect.
FNC, $n = 3$ (white bars), FNPC cultured in the absence of ALLO, $n = 5$ (black bars) and FNPC cultured in the presence of 50 nM ALLO for 24 hrs, $n = 5$ (grey bars) or with 10 mM N-acetyl cysteine (NAC) (hatched bars) were treated for 1 hr with the indicated concentrations of H_2O_2, labelled with Annexin V-FITC and analysed by flow cytometry. The effect of the indicated concentrations of H_2O_2 on Annexin V-FITC is expressed as percentage of the value of non-treated (NT) cells. The differences in Annexin V-FITC fluorescence between FNC (white bars) and FNPC (black bars) are significant at the H_2O_2 concentrations of 2.5 and 5.0 mM (*a, $P < 0.05$). The effect of ALLO (grey bars) and NAC (10 mM) (hatched bars) on Annexin V-FITC fluorescence in FNPC *versus* untreated FNPC (black bars) was statistically significant at all concentrations of H_2O_2 (**b $P < 0.01$; *b $P < 0.05$).

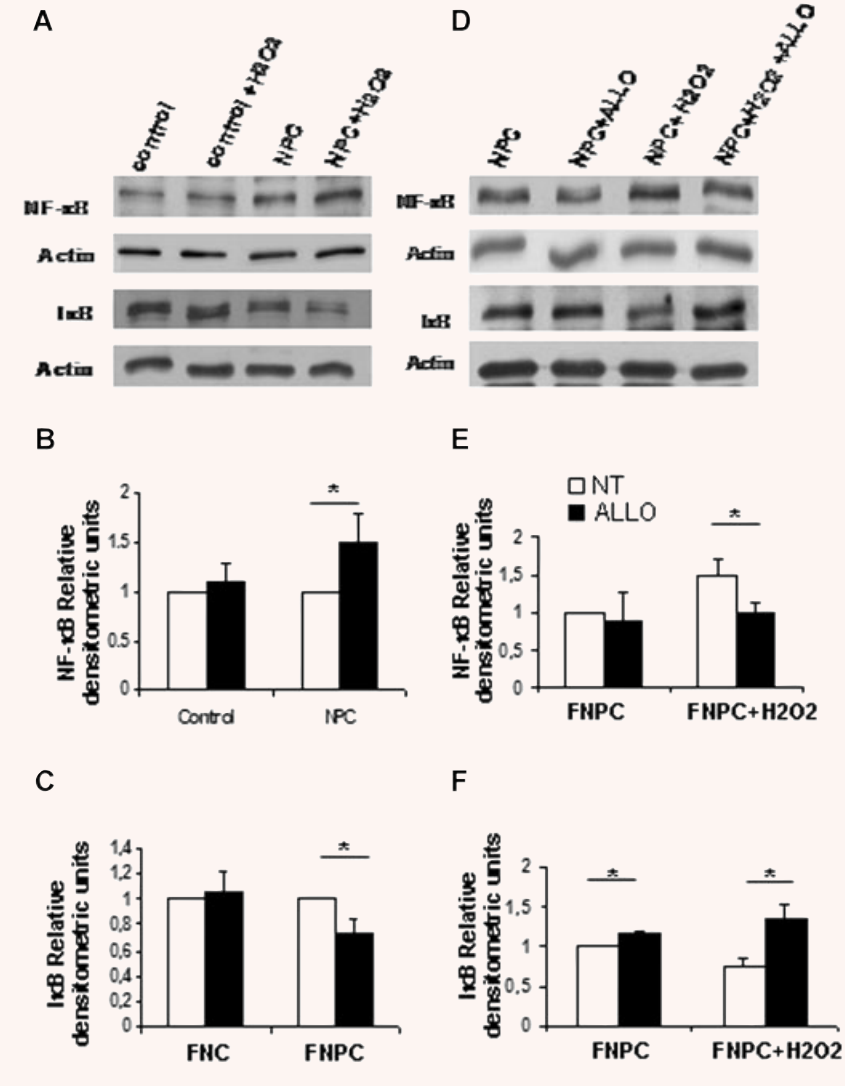

Fig. 7 NF-kB and IκB in FNC *versus* FNPC: effect of ALLO
FNC ($n = 3$) and FNPC ($n = 4$) were treated with H_2O_2 2.5 mM for 1 hr. Nuclear or cytosolic proteins were purified and the levels of NF-kB, IkB were estimated by Western blot. (**A**) Representative Western blot analysis of the nuclear expression of NF-kB and cytosolic expression of IkB. (**B**) The intensity of NF-kB signals were normalized against actin and expressed as a percentage of the concentrations found in untreated cells (NT). Data are means ± S.D. of 3 independent experiments (* $P < 0.05$). (**C**) The intensity of IkB signals were normalized against actin and expressed as a percentage of the concentrations found in untreated cells (NT). Data are means ± S.D. of 3 independent experiments (* $P < 0.05$). FNPC ($n = 4$) in the absence (NT) or presence of ALLO (50 nM for 24 hrs) were treated with H_2O_2 2.5 mM for 1 hr, nuclear or cytosolic proteins were purified and the levels of NF-kB and IkB were determined by Western blot. (**D**) Representative Western blot analysis of the nuclear expression of NF-kB and cytosolic expression of IkB. (**E**) The intensity of NF-kB signal in NPC cells was normalized against actin and expressed as a percentage of the concentrations found in untreated cells (NT, white bars). Data are means ± S.D. of 3 independent experiments (* $P < 0.05$). (**F**) The intensity of signal IkB in NPC cells was normalized against actin and expressed as a percentage of the concentrations found in untreated cells (NT, white bars). Data are means ± S.D. of 3 independent experiments (* $P < 0.05$).

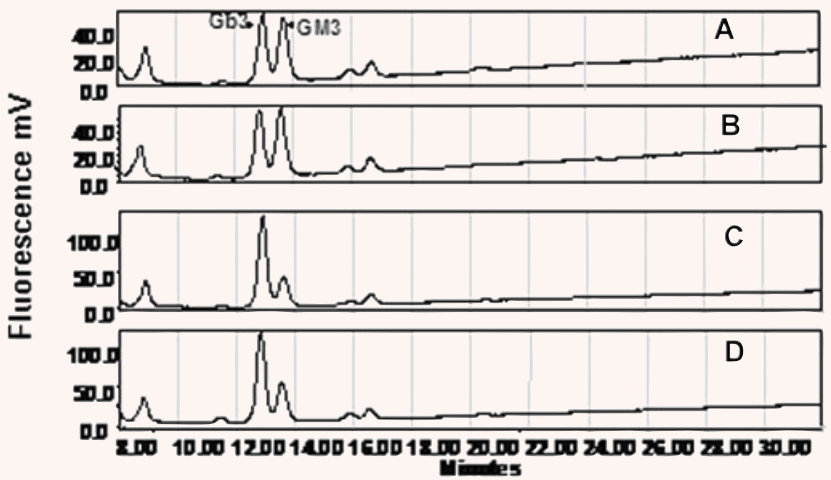

Fig. 8 HLPC analysis of GSL in FNPC. Representative GSLs profiles of fibroblasts from 2 different NPC patients, cultured in the absence of ALLO (**A** and **C**) or in the presence of ALLO (50 nM, 24 hrs) (**B** and **D**). ALLO treatment did not induce a significant reduction of GSLs.

Fibroblasts from NPC patients may be exposed to chronic oxidative stress that renders them more sensitive to peroxide-induced apoptosis. These results are similar to those observed in fibroblasts from Gaucher disease patients [11], suggesting a common pathogenic mechanism. The increased sensitivity to peroxide-induced apoptosis in fibroblasts from NPC patients might be mediated at least in part by activation of the NF-κB pathway.

Neurosteroidogenic abnormalities have been found in NPC disease [14]. The mechanism by which neurosteroids provide neuroprotection is not understood, and it may involve both genomic and non-genomic actions. Non-genomic actions of neurosteroids mediated through neurotransmitter receptors have been studied extensively. Reduced progesterone derivatives such as ALLO can activate $GABA_A$ receptors [29, 30], and some effects of ALLO on NPC mice appear to be mediated through $GABA_A$ receptors [14]. However, the synthetic enantiomer of ALLO, ent-ALLO, provided identical effects as natural ALLO in NPC1 mice [15], suggesting that some effects of ALLO in the NPC mice may not be mediated by $GABA_A$ receptors.

Our studies show that low nanomolar concentrations of ALLO reduced the intracellular levels of ROS in fibroblasts from NPC patients, and in primary fibroblast cultures and neuronal SHSY5Y cells in which NPC1 expression was knocked down. ALLO treatment also reduced lipid peroxidation and almost completely reversed peroxide-induced apoptosis in NPC fibroblasts. These effects were not associated with a reduction in GSL or cholesterol accumulation, suggesting that ALLO might act directly on the intracellular redox status rather than by changes in intracellular GSL or cholesterol accumulation.

ALLO activates the pregnane-X receptor, PXR, at concentrations of 10–50 μM [15]. Although PXR is expressed in human fibroblasts (data not shown), it is unlikely that ALLO mediates its effects through this receptor in fibroblasts since nanomolar concentrations of ALLO maximally reduced ROS concentrations and prevented peroxide induced apoptosis. Hence, it is unlikely that the nuclear PXR is involved in the effect of ALLO on reducing oxidative stress in NPC fibroblasts.

In addition to the classical nuclear actions, steroids can also regulate cell signalling phosphorylation cascades and exert actions initiated at the membrane, which in most cases are rapid [31–35]. Our results showed a very rapid reduction (30 min.) of the intracellular levels of ROS in FNPC and in NPC neuronal model (SHSY5Y NPC1 knock-down cells) exposed to 50 nM of ALLO, suggesting that ALLO might regulate the intracellular redox state though a non-genomic mechanism. Furthermore, a very similar effect on ROS levels was observed when both FNPC and SHSY5Y NPC1 knock-down cells were exposed to ent ALLO. Because natural ALLO and *ent*-ALLO interact with membranes in an identical manner [16, 17], it is possible that the effects of these compounds on ROS levels are mediated through direct steroid–membrane interactions.

The antioxidant effects of estrogens seem to be associated with their intrinsic free radical scavenger activity due to the presence of a phenolic-A ring (estrogens) [36] or a C3 hydroxyl group (estradiol) [37]. Although ALLO does not contain a phenolic A ring, a similar mechanism could be hypothesized. However, our data suggest that ALLO does not act as a free radical scavenger.

In the light of our results, it is possible to speculate that ALLO might reduce the intracellular concentrations of ROS by increasing CAT activity. The molecular mechanism by which ALLO restores CAT activity in FNPC needs to be further investigated. However, the rapid reduction of ROS levels suggests that ALLO would not mediate its effects on CAT activity through a transcriptional activation.

ALLO might protect NPC cells from peroxide-induced apoptosis through the inhibition of NF-κB signalling. In NPC mice, ALLO suppresses the expression of TNF-α [13], a cytokine that activates NF-κB [38]. Taken together, these data suggest that the effects of ALLO on NF-κB involve multiple pathways.

Recently, a reduction of ALLO concentrations was found in prefrontal cortex and in plasma of AD patients [39, 40]. Results obtain in the mouse model of AD suggest that ALLO may be a

promising therapeutic strategy for treatment of AD [41, 42]. Because the role of oxidative stress in the pathophysiology of AD has been documented extensively [9, 43], our results suggest that ALLO might act beneficially through similar mechanisms to reduce oxidative damage in both NPC and AD.

Successful treatment of NPC mice was reported with a single injection of ALLO at post-natal day 7 (P7) [14, 44]. The long-term effects of this single treatment suggest that ALLO might be involved in neurodevelopmental processes. Our data suggest that ALLO may protect cells from oxidative damage and from peroxide-induced apoptosis at critical times in neurodevelopment.

Thus, we have shown that NPC1 deficiency causes an imbalance in the intracellular redox state, which could be restored by ALLO treatment *in vitro*. These findings suggest that oxidative stress might play a role in the pathophysiology of NPC disease and that ALLO could be beneficial in the treatment of the disease, at least in part, due to its ability to restore the intracellular redox state. In addition, our results demonstrate that the effects of ALLO are pleiotropic and it could act by different mechanisms and on different cell types including cells outside the central nervous system (CNS). As many of these processes are similar among neurodegenerative diseases, our results suggest that other brain diseases may benefit from similar treatments with neurosteroids.

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