Research paper

Hydrogen peroxide modifies both activity and isoforms of acetylcholinesterase in human neuroblastoma SH-SY5Y cells

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A B S T R A C T

The involvement of cholinergic system and the reactive oxygen species (ROS) in the pathogenesis of some degenerative diseases has been widely reported; however, the specific impact of hydrogen peroxide (H_2O_2) on the acetylcholinesterase (AChE) activity as well as AChE isoform levels has not been clearly established. Hence, the purpose of present study is to clarify whether H_2O_2 alters these parameters.

Human neuroblastoma SH-SY5Y cells were treated with H_2O_2 (1–1000 µM) for 24 h and AChE activity and AChE and cytochrome c levels were evaluated. AChE activity was strongly increased from 1 µM to 1000 µM of H_2O_2. The results of the kinetic study showed that H_2O_2 affected Vmax but not Km; and also that H_2O_2 changed the sigmoid kinetic observed in control samples to hyperbolic kinetic. Thus, results suggest that H_2O_2 acts as an allosteric activators. In addition, H_2O_2, (100–1000 µM) reduced the total AChE content and modified its isoform profile (mainly 50-, 70-, and 132-kDa)·H_2O_2 from 100 µM to 1000 µM induced cytochrome c release confirming cell death by apoptosis. All these results together suggest: a) the involvement of oxidative stress in the imbalance of AChE; and b) treatment with antioxidant agents may be a suitable strategy to protect cholinergic system alterations promoted by oxidative stress.

1. Introduction

Acetylcholinesterase (AChE) (EC 3.1.1.7) is the enzyme responsible for the termination of cholinergic neurotransmission [1]. Several neurological disorders, chiefly Alzheimer disease (AD), are associated with abnormal expression and activity of this enzyme [2,3]. In fact, AChE inhibitors (AChEIs) are used as gold standard treatment in Alzheimer disease (AD) [4]. Although the true benefit of these drugs is currently coming into question [5].

Alternative splicing gives rise to three isoforms of AChE from a single gene. Such isoforms differ in their C-termini [6], leading to different post-translational modifications [7]. Nevertheless, all of them retain the capacity to hydrolyze Ach [8]. These isoforms are known as AChE-R, AChE-E and AChE-S [9]. AChE-E is found in erythrocytes, while AChE-R and AChE-S are the splice variants present in the brain. AChE-S appears as monomers (G1) and also as dimers (G2) or tetramers (G4) formed by assembly with a proline-rich membrane anchor [10]. The AChE-R is related to external stressors [9] and its soluble monomeric subunit seems to be able to associate to form complexes without disulphide bounds [11].

Although reactive oxygen species (ROS) are continuously produced in the cells [12], it is widely accepted that oxidative stress plays an important role in many chronic disease, including neurodegenerative diseases. In fact, aging, one of the main risk factors of these diseases, is associated with ROS accumulation [13]. Moreover, ROS increase is detected in the early stages of such diseases, even when others characteristic features remain unchanged, suggesting that oxidative stress is implicated in their etiology [14]. For example, oxidative stress has been found in AD models even when Aβ levels remained unchanged [15]. Regarding AD, both, oxidative stress and AChE have been linked to Aβ generation.

Effect of H_2O_2 on AChE has been studied in different models. Thus, Schallreuter et al. [16] found that H_2O_2 a high concentrations, which is characteristic of epidermal vitiligo disease, regulates AChE activity in a dose-dependent manner, via oxidation of methionine, cysteine and tryptophan all residues closely related to the active centre. Also, Zhang et al. [17] observed a H_2O_2-dependent increase of AChE expression via transcriptional JNK; and Molochkina et al. [18] showed that H_2O_2

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modifies AChE activity by modification of the cellular membrane. Despite all of these works, no link between oxidative stress and AChE has been identified in human brain cells. In this context, it would be interesting to know the effect of ROS on cholinergic system markers from undifferentiated SH-SY5Y neuroblastoma cells; because, although these cells are not specifically cholinergic, they present AChE enzyme as has been previously demonstrated by Filograna et al. [19]. Although there are some studies about the action of pro-oxidant substances on AChE of different cell lines, to the best of our knowledge the direct effect of oxidative stress on the levels of AChE and AChE activity in human brain cells remains unclear. In the present study it was hypothesized that H_2O_2 produces a specific effect on AChE in the human neuroblastoma SH-SY5Y cell line, modifying the levels and isoform profile of this enzyme.

H_2O_2 is particularly interesting because: a) it is the most stable ROS [20]; b) it passes through cell membranes [21]; and c) it does not act just as an extracellular messenger but also as an intercellular one, causing alteration of several proteins [22].

The present study in SH-SY5Y human neuroblastoma cell line aims to determine the effect of H_2O_2 on AChE activity and isoform profiles.

2. Materials and methods

2.1. Reagents/Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA, and penicillin/streptomycin mixture were purchased from GIBCO–BRL (Grand Island, NA, USA)·H_2O_2, acetylthiocholine iodide, 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB), aprotinin, leupeptin and phenyl-methane-sulfonyl fluoride (PMSF) and Triton X-100 were obtained from Sigma Chemical Co.(St. Louis, MO, USA). Anti-acetylcholinesterase antibody (N-19) (sc-6431), anti-cytochrome c (sc-8385), and anti β-actin antibody (sc-47778) were supplied by Santa Cruz Biotechnology, Quimigen (Madrid, Spain). PVDF membrane and ECL select were purchased from GE Healthcare (Madrid, Spain). Other chemicals were reactive grade products from Merck (Darmstadt, Germany).

2.2. Cell culture
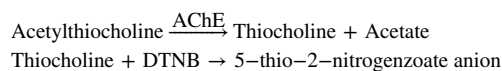
Human SH-SY5Y (ATCC CRL-2266) neuroblastoma cells were cultured in DMEM with 10% (v/v) FBS and 100 U/mL penicillin/ streptomycin at 37 °C in a 5% CO_2 and 95% air humidified atmosphere. When cells reached 80–90% confluence were sub-cultured on 100 mm^2 culture dishes with 10% (v/v) FBS. 24 h later cells were changed to media with 1% FBS and treated with or without H_2O_2 (1–1000 µM) during 24 h·H_2O_2 was freshly prepared from 30% stock solution prior to each experiment. The control cells were kept in media without H_2O_2. The experiments were carried out between 3 and 15 passages.

2.3. Estimation of protein

The protein concentration was determined in cell extracts according to Bradford method [23], using bovine serum albumin as standard. The cells were lysed with the buffer used to measure the AChE activity, pelleted and centrifuged (Centrifuge 5804R, Eppendorf, Hamburg, Germany) at 13,0000g during 15 min. The protein content was determined on supernatants.

2.4. Determination of activity and kinetic parameters of AChE

AChE activity was determined by Ellman's colorimetric method [24] based on the following reactions:

$$Acetylthiocholine \xrightarrow{AChE} Thiocholine + Acetate$$
$$Thiocholine + DTNB \rightarrow 5-thio-2-nitrogenzoate\ anion$$

In these experiments we have used SH-SY5Y extracts as AChE source. Cells were seeded in 100 mm^2 dishes (2×10^6 cells) and treated during 24 h with the indicated H_2O_2 concentrations. The cells extracts (enzyme source) were prepared homogenizing the cells with 0.1 M KH_2PO_4/K_2HPO_4 (pH 8.0), containing 0.1% (v/v) Triton X-100, and centrifuging at 13,000g for 5 min. Supernatants (50 µl) were mixed with 4 mM of DTNB, increasing concentrations of Acetylthiocholine (50–1000 µM), and phosphate buffer 0.1 M, pH=8. The final volume was 205 µl. The yellow product of this reaction was measured spectroscopically at 412 nm. (LT-4000, Microplate Reader, Labtech International Ltd, United Kingdom), every minute during 10 min. Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined by Lineweaver– Burk plot method [25], using the inverse of activity (DO/min/mg protein) and the inverse of substrate concentration (µM). The activity was expressed in OD/m/mg protein.

2.5. Effect of H_2O_2 on AChE activity from control cell extracts

In this experiment cell culture was performed slightly different. The cells were seeded in 100 mm^2 dishes (2×10^6 cells) with 10% FBS media for 24 h. After that time the plates were not treated with H_2O_2. Instead of that, they were kept with 1% FBS media during 24 h, as we did with the control cells of the rest of experiments. The cells extracts were obtained as described above (Section 1.4), homogenizing the cells with the buffer and centrifuging at 13,000g for 5 min. The treatment was carried out once the supernatants were isolated·H_2O_2 (1, 400 and 1000 µM final concentration) was mixed with the cell extract and incubated for 0, 15 or 30 min. In this experiment the control was the extract of the cells without adding H_2O_2.

Afterwards, the enzymatic AChE activity was measured as indicate above (section 1.4). Finally, AChE activity defined as punctual velocity (Vp) was normalized to the amount of protein of the extracts and expressed as OD/min/mg protein.

2.6. AChE and cytochrome c levels

The protein content was assayed by Western Blot following Garcimartín et al. protocol [26]. SH-SY5Y cells were plated on 100 mm dishes and treated with H_2O_2 (1–000 µM) for 24 h. To prepare the samples for AChE analyses the cells were lysed with buffer Tris-HCl (pH 7.4), 1% Triton X-100, 10 mM EDTA, 50 mM NaCl and enriched with aprotinin (2 µg/mL), leupeptin (5 µg/mL) and PMSF (1 mM) as protease inhibitors. After that they were centrifuged at 13,000g for 5 min at 4 °C to remove the nuclei and cellular debris (Centrifuge 5804R, Eppendorf, Hamburg, Germany). The samples were manipulated in the same way that the SH-SY5Y cells. On the other hand, to prepare the cytochrome c sample subcellular fractionation was needed [26]. The cells were harvested in hypotonic buffer (10 mM Tris-HCl (pH 7.4), 1 mM sodium vanadate and protease inhibitors) and incubated on ice. The lysates were centrifuged at 800g for 5 min at 4 °C to remove the nuclei and cellular debris. The resulting supernatants were further centrifuged at 15,000g for 15 min at 4 °C to obtain the supernatant fraction (cytosol). Before starting, 5x-Loading buffer (0.5 M Tris-HCl (pH 6.8), 20% v/v glycerol, 10% w/v SDS, 5 mM β-mercaptoethanol and 0.01% bromophenol blue) was added to each sample and incubated for 5 min at 95 °C. Equal amounts of protein (30 µg) were separated at 150 V in 10% (v/v) polyacrylamide gel (SDS–PAGE) and, after migration, transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Madrid, Spain) at 400 mA for 1 h, at 4 °C. All membranes were blocked by 5% (w/v) non-fat dry milk for 1 h at room temperature. For immunodetection, membranes were incubated overnight at 4 °C with the primary antibody, anti-AChE (1/1000) or anti-cytochrome c (1/1000), followed by incubation with peroxide-conjugated second-

ary antibodies for 1 h at room temperature (Santa Cruz Biotechnology, Quimigen, Madrid, Spain). Blots were developed by enhanced chemiluminescence (ECL select; GE Healthcare, Madrid, Spain) according to the manufacturer's instructions. Anti β-actin antibody (1/2000) was used as loading control.

2.7. Quantification of protein levels and AChE isoforms

The quantification of the protein levels was made using Image Quant 5.0 software. Each band was measured individually, removing the background signals, and expressed as densitometry unit respect to beta-actin (densitometry/beta actin). To know the content of the whole AChE protein, the corrected data of all bands were added up and considered as the 100% of total AChE protein. The specific contribution of each band to global AChE content was expressed as the percentage of each band (band densitometry × 100/ total densitometry AChE protein).

2.8. Principal component analysis

PCA test was applied to understand possible associations between the different bands detected in western blot, which represent the isoforms of AChE, and the kinetic parameters. Ten different variables were tested, seven, related to percentage of isoforms (132-, 100-, 70-, 65-, 55-, 50-, 40-kDa bands), two about kinetic parameters (Vmax and km). PCA create new variables (principal components (PCs)) containing the initial variables which correlate among them. Data number included in PCA was higher than five times (> 5x) the number of variables.

2.9. Statistical analysis

Data were expressed as mean ± SEM from two independent experiments using different cultures. Each experiment was performed at least in triplicate with different cell batches (total 6–12 measurement/ condition). Statistical analyses were performed using SigmaPlot 11.0 software. Data were tested by One Way ANOVA followed by the Bonferroni test. Differences were considered significant at $P < 0.05$. SPSS version 19.0 statistical analysis package (SPSS, Inc., Chicago, IL, USA) was used for the PCA test and linear correlations.

3. Results

3.1. Effect of H_2O_2 on AChE activity

As some neurodegenerative diseases have been related with changes in AChE activity along with an increase in oxidative stress, we measured the AChE activity from SH-SY5Y cells which were used as enzyme source. The activity was test in control cells (untreated cells) and in cells after 24 h treatment with H_2O_2. The H_2O_2 (1–1000 μM) promoted an increase in AChE activity. The highest effect was produced at 1 μM and it was kept until 1000 μM (Fig. 1).

Such an effect on AChE activity could be caused by direct action of the H_2O_2 on the structure of the enzyme or by an alteration of the AChE levels, and therefore three different experiments were carried out to ascertain the mechanism involved: a) AChE kinetic parameters evaluated in control cells and after treating the cells during 24 h with different concentrations of H_2O_2; b) AChE activity after the addition of H_2O_2 to control cell extracts; and c) measure of AChE levels by western blot analysis in control cells and in cells subsequent to the 24 h treatment with H_2O_2.

3.2. Action of H_2O_2 on kinetic parameters of AChE

The plot of AChE activity vs acetylthiocholine (substrate) concentration allowed us to further characterize the enzyme and helped to
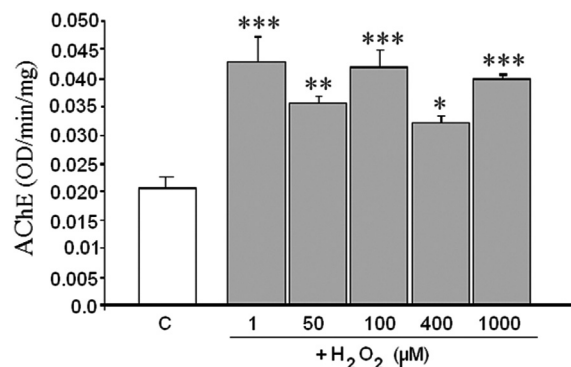

Fig. 1. Effect of different concentrations of H_2O_2 on AChE activity from SH-SY5Y as enzyme source. Cells were treated during 24 h with 1, 50, 100, 400 and 1000 μM of H_2O_2. Control condition (C) is referred to untreated cells. AChE activity was determined by the Ellman's method, using acetylthiocholine (250 μM), and expressed in OD/m/mg protein. Results are means ± SEM of cells from three different cultures each one performed in triplicate. (*) = statistical signification with respect to control. (*) = p < 0.05, (**) = p < 0.01 and (***) = p < 0.001.

know the enzyme behavior (Fig. 2).

The AChE activity of control cells (kept 24 h in 1%FBS media without any treatment) and those treated with 1 μM of H_2O_2 showed an evident sigmoid adjustment with a Hill coefficients of 3.0 ± 0.4 (control cells) and 2.5 ± 1.5 (1 μM H_2O_2) (Fig. 2a). These Hill coefficient values are characteristic of allosteric enzymes and suggest that the enzyme has 2–3 identical sites for the substrate probably because it was composed of 2–3 subunits. The values of kinetic parameters (Vmax, Km, and Hill scope) are displayed on Table 1. When the cells were treated with higher concentrations of H_2O_2 (50 μM to 1000 μM) the sigmoid behavior turned into a hyperbolic one (Fig. 2b). Furthermore, H_2O_2 treatment affected the Vmax, but not Km of AChE, respect to control (Table 1).

3.3. Effect of H_2O_2 on AChE activity in control cell extracts

The effect of H_2O_2 on AChE activity was measured in the extracts obtained from control cells in order to know the way in which H_2O_2 affects AChE activity on the enzyme without any possible additional effect mediated by the cells. With this experiment we were able to discount the effect of H_2O_2 on AChE levels, and the effect of other substances generated inside the cell as a consequence of the toxic action of H_2O_2 (e.g. other ROS or Aβ among others). At the beginning and 15 min after the addition of H_2O_2 to control cell extracts there was non-significant change in AChE activity (data not shown) respect to control. However, after 30 min of H_2O_2 exposure, the enzyme activity increased, confirming the direct effect of H_2O_2 on the structure of the enzyme. Furthermore, this experiment indicated that the effect was not immediate, but H_2O_2 needed time to induce it. Moreover, the effect displayed by H_2O_2 in this case, and in contrast to the previous experiment using treated cells (Fig. 1), was dose-dependent (Fig. 3). This difference detected between both experiments suggested that other mechanism could be involved when the cells are treated with H_2O_2 apart from the one demonstrated.

3.4. Impact of H_2O_2 on AChE levels

Western blot analysis allowed us to know the levels and isoforms profile of AChE from control undifferentiated SH-SY5Y cells and their possible changes after H_2O_2 treatment. Control samples, without any treatment, presented several bands weights of 132-, 100-, 70-, 65-, 55-, 50- and 40-kDa (Fig. 4a)·H_2O_2 induced a clear dose-dependent decrease in the levels of AChE bands (Fig. 4b) judging by the lower bands density in the samples from treated cells.

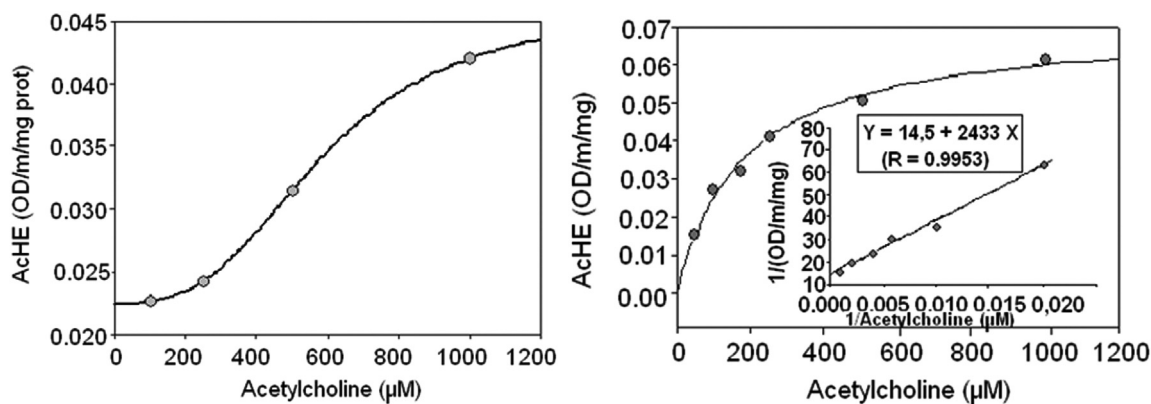The densitometry values of each band (expressed as arbitrary

Fig. 2. Representation of acetylcholinesterase (AChE) activity vs. acetylthiocholine concentration. Kinetic analysis was performed by the Ellman's method using different concentration of substrate and SH-SY5Y extracts as enzyme source. Enzyme behavior was obtained using the SigmaPlot analysis which evaluated whether the data are adjustable to sigmoidal or hyperbolic adjusts. **(a)** Sigmoidal adjustments typical of control cells and those treated with 1 µM of H_2O_2; and **(b)** hyperbolic adjustment characteristic of cells treated with H_2O_2 (50–1000 µM). Results are means ± SEM of cell from two different cultures, each one performed in triplicate.

Table 1
Kinetic parameters of AChE in SH-SY5Y cells after 24 h treatment with H_2O_2.

+ H_2O_2 (µM)	Vmax OD/m/mg	Km (µM)	Curve type	Hill Scope
Control	0.039 ± 0.0011	135 ± 20	Sigmoidal	2.95 ± 0.4
1	0.071 ± 0.0028 P < 0.001	232 ± 31 (ns)	Sigmoidal	2.50 ± 1.5
50	0.061 ± 0.004 P < 0.001	195 ± 25 (ns)	Hyperbola	–
100	0.075 ± 0.0032 P < 0.001	218 ± 14 (ns)	Hyperbola	–
400	0.084 ± 0.006 P < 0.01	280 ± 11 (ns)	Hyperbola	–
1000	0.062 ± 0.004 P < 0.001	172 ± 24 (ns)	Hyperbola	–

* Control condition is referred to untreated cells. Results were obtained from the evaluation with the SigmaPlot and represent data from two different cultures each one performed in triplicate. ns: non-significance.
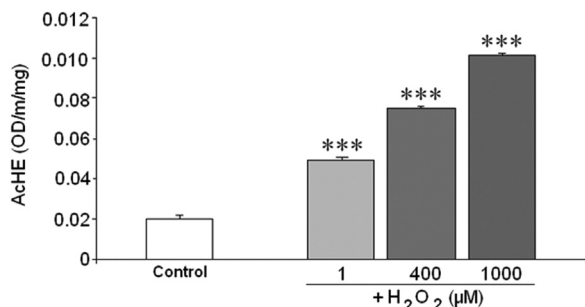

Fig. 3. Detection of acetylcholinesterase (AChE) activity of control cell extracts from SH-SY5Y cells as enzyme source treated or not with H_2O_2 (1, 400 and 1000 µM) during 30 min·H_2O_2 was added to the AChE extract previously obtained from untreated cells. AChE activity was measured by the Ellman's methods using acetylthiocholine (250 µM) as substrate. ***P < 0.001.

densitometry unit respect to β-actin), as well as the total levels obtained by adding the densitometry values of each band from each condition are displayed in Fig. 4c. In this figure it can be observed how the total AChE levels were significantly reduced by H_2O_2 from 100 to 1000 µM respect to control sample·H_2O_2 at 50 µM significantly decreased the 50-kDa band. The 132- and 70-kDa bands decreased from 100 µM of H_2O_2 disappearing at 1000 µM (P < 0.001 vs. control). Oppositely, H_2O_2 at 1000 µM increased the 55-kDa bands (P < 0.01). The statistical significations of individual bands are displayed in the table inserted in Fig. 4c.

3.5. Contribution of isoforms to the total AChE content

The contribution of each particular isoform to the total AChE protein would give a clear idea of the isoform profile resulting from

the alternative splicing of this enzyme. The percentages of the isoforms are presented in Table 2. The 50-kDa band seemed the more susceptible to H_2O_2 treatment since it changed respect to control sample from 50 µM. The other bands were not modified until 400 µM (100-kDa) and 1000 µM of H_2O_2 (132-kDa). In fact, such treatment with H_2O_2 (1000 µM) clearly modified the balance between isoforms. Bands at 100- and 55-kDa significantly increased (5.3 and 4.95 times, respectively) their contribution to total protein compared with the control cells, while 50-kDa band decreased (4.6 times). Besides, the 132- and 70-kDa bands disappeared.

3.6. PCA results

Principal component analysis (PCA) was conducted to ascertain possible relationships between bands, together with the link between AChE activity and isoform profile. The two isoforms present in the brain (AChE-R and AChE-S) could appear as G1 forms, but also associated as G2 or higher structures, and hence some of the bands that we detected in the western blot are likely to be related to one another (Table 3, Fig. 5a and b).

Nine different variables were evaluated: the contribution (%) of the seven different isoforms (132-, 100-, 70-, 65-, 55-, 50- and 40-kDa), and two related to the kinetic parameters (Vmax and Km). The PCA created two components which explained the 79.01% of the total variance in the data set (Fig. 5a). First component (PC-1) accounted for 64.06% of the variance. It correlated strongly and positively with 132-, 70- and 50-kDa bands (see lower right quadrant of the plot) and their values indicated in Table 3 were close to 1. On the other hand, there were 100-, 65- and 55- kDa bands, with values close to −1, located on the left side of the plot (Fig. 5a), which correlated negatively with the other bands.

Second component (PC-2) explained 14.96% of the total variance. It correlated positively with both kinetic parameters, Km and Vmax, and also with the 40-kDa band, appearing at the top of the plot with the 40-kDa band alone in the middle of the plot (Fig. 5a). Summarizing, the PCA scatter plot divides the different bands in two groups of three bands each, appearing the 40-kDa band in the middle of both. Moreover, the kinetic parameters seemed to be more related to the 132-, 70- and 50-kDa bands. The PCA scatter plot (Fig. 5b) indicates the position of the studied groups in a graph compiled with two components (PC-1 and PC-2). It showed how the control cells were located on the positive part of PC-1 but the negative part of PC-2, while with growing H_2O_2 concentrations (from 1 µM to 400 µM), PC-2 positive values, shifted to more positive positions. Finally, the position of the treatment with 1000 µM of H_2O_2 was completely different from the other treatments, situated on the negative values of PC-1.
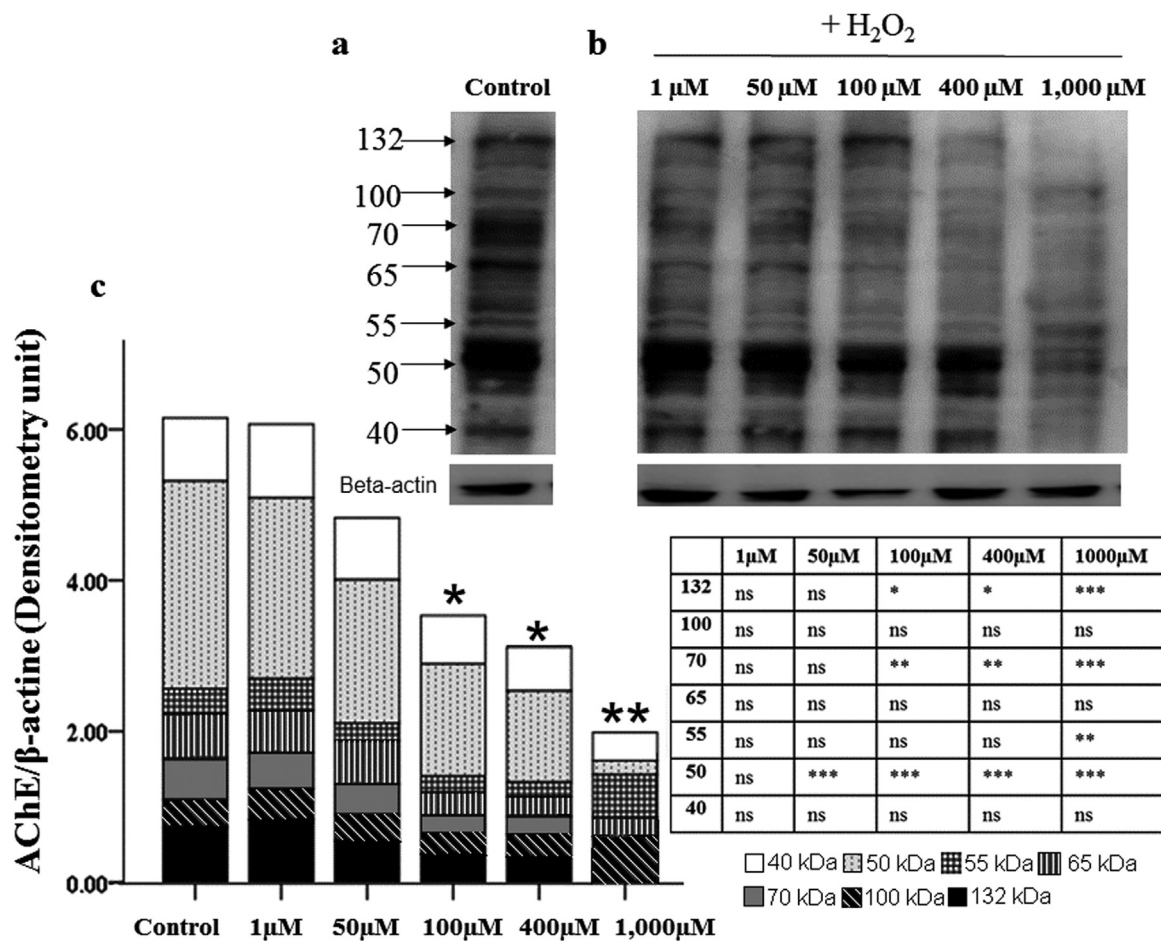
Fig. 4. Immunoblots of acetylcholinesterase (AChE) using SH-SY5Y cells extracts as protein source prepared after 24 h treatment with H$_2$O$_2$. Control condition (C) is referred to untreated cells. **(a)** Immunoblots of control cells with different bands due to the characteristic alternative splicing of AChE gene. **(b)** Immunoblots of cells treated with increasing concentrations of H$_2$O$_2$ (1, 50, 100, 400 and 1000 μM). **(c)** Quantification of the immunoblots. The results were normalized against beta actin. All isoforms are represented in a stacked column chart from the heaviest (132-kDa) to the smallest (40-kDa). Results of three different cultures each one performed by duplicated. Statistical signification is shown in the table (Bonferroni test). ns = non-significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 2
Contribution of isoforms to the total AChE content in SH-SY5Y cells after 24 h treatment with H$_2$O$_2$.

Isoform	+ H$_2$O$_2$ (μM)					
	Control	1	50	100	400	1000
132	11.19 ± 0.4	13.20 ± 3.3 ns	13.71 ± 2.6 ns	10.92 ± 2.1 ns	11.00 ± 1.3 ns	0.00 P < 0.001
100	5.68 ± 0.5	6.50 ± 1.3 ns	6.73 ± 2.3 ns	7.38 ± 1.0 ns	9.32 ± 0.8 P = 0.5	30.45 ± 3.4 P < 0.001
70	8.25 ± 1.0	7.84 ± 1.0 ns	8.06 ± 1.2 ns	6.61 ± 2.1 ns	7.69 ± 0.4 ns	0.00 P < 0.001
65	8.68 ± 3.1	8.83 ± 1.0 ns	11.13 ± 2.7 ns	8.96 ± 0.3 ns	8.44 ± 1.1 ns	14.5 ± 2.1 ns
55	5.15 ± 0.3	6.84 ± 2.7 ns	4.50 ± 0.9 ns	6.51 ± 1.3 ns	6.25 ± 1.4 ns	25.51 ± 3.3 P < 0.001
50	47.05 ± 1.6	41.54 ± 4.1 ns	39.81 ± 0.5 P = 0.03	40.68 ± 1.0 P = 0.05	38.73 ± 1.0 P = 0.011	10.25 ± 1.9 P < 0.001
40	9.21 ± 7.7	9.22 ± 7.3 ns	10.09 ± 8.1 ns	12.72 ± 10.6 ns	12.76 ± 10.7 ns	14.07 ± 11.9 ns

† Control condition is referred to untreated cells. The corrected data (densitometry unit respect to beta-actin) of all bands were added up and considered as the 100% of total AChE protein. The specific contribution of each band to global AChE content was expressed as the percentage of each band (band densitometry x 100/ total densitometry AChE protein). ns: non-significance.

3.7. Effect of H$_2$O$_2$ on cytochrome c release

In order to establish the relation between the effect of H$_2$O$_2$, AChE and apoptosis, the levels of cytochrome c in cytosol were tested by immunoblot. The presence of cytochrome c in the cytosol increased in parallel to H$_2$O$_2$ concentrations tested, being significant for concentrations of H$_2$O$_2$ ≥ 100 μM (Fig. 6).

4. Discussion

This paper offers novel information about the H$_2$O$_2$ (ROS) mechanism operating on AChE from SH-SY5Y human neuroblastoma cells. The study has the strength of testing five increasing concentrations of H$_2$O$_2$ and assessing both the activity and the protein levels of AChE as well as cytochrome c release. Present findings are interesting given the presence of oxidative stress, as well as the implication of AChE in the early stages of neurodegenerative diseases [27,28].

Table 3
Rotated component matrix for the analysis of the relation between AChE isoforms (%) and kinetic parameters (Vmax and km).

Variable	PC-1	PC-2
Vmax	0.506	0.796
Km	0.658	0.630
132 (%)	0.925	−0.085
100 (%)	−0.957	0.184
70 (%)	0.953	−0.104
65 (%)	−0.748	0.074
55 (%)	−0.968	0.101
50 (%)	0.939	−0.268
40 (%)	−0.137	0.420

‡ Factors were extracted with the principal component analysis (PCA) using the Varimax rotation. PC, principal component.
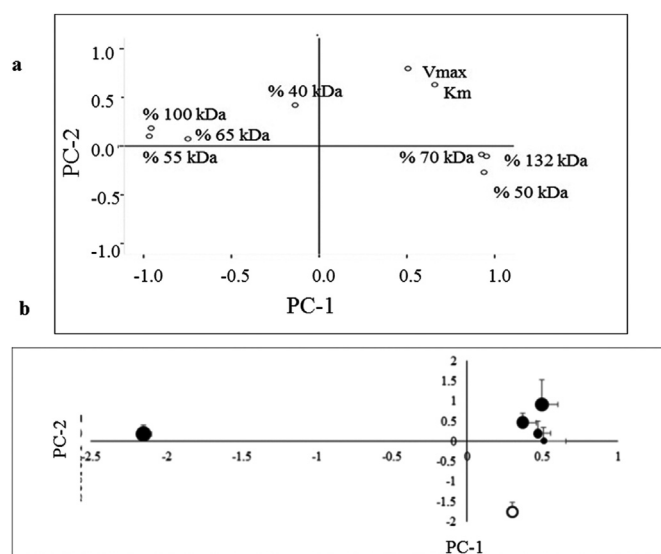
Fig. 5. Principal component analysis (PCA) results. **(a)** Loading PCA plot for nine variables: the contribution (%) of the AChE seven isoforms (132-, 100-, 70-, 65-, 55-,50- and 40-kDa), and the kinetic parameters (Vmax and Km). Dot lines at 0 in the PC-1, and PC-2 scales mean no contribution of each variable to the specific PC. This figure shows the different correlations between the different variables considered taking into account the two PCA components. **(b)** Scatter plot using nine variables: the contribution (%) of the AChE seven isoforms (132-, 100-, 70-, 65-, 55-, 50- and 40-kDa), and the kinetic parameters (Vmax and Km). PC-1 and PC-2 accounted for 64.06% and 14.96% of the variance, respectively. Dot lines at 0 in the PC-1 and PC-2 scales means no explanation by the specific PC to the variability found in each treatment. ○ Control cells; ● 1 μM H₂O₂; ● 50 μM H₂O₂; ● 100 μM H₂O₂; ● 400 μM H₂O₂; ● 1000 μM H₂O₂.

4.1. H₂O₂ induced AChE activation

The present results show the activation of AChE when the cells were treated with H₂O₂. Such activation, triggered by oxidative stress, would imply an additional damage to the neurons beside the neuronal death. Activator effect of oxidative stress promoted by H₂O₂ or other pro-oxidant substances on AChE has been previously found by several authors in different cell lines [17,18,29,30]. Jiang et al. [30] detected an increase in AChE activity after H₂O₂ insult in PC12 cells. These authors attributed the increase in AChE activity to oxidative stress because Glutathione, which is involved in cellular redox homeostasis, inhibited the increase of AChE activity. These results agree with ours; however, these authors did not study the mechanism by which oxidative stress induces AChE activation. Molochkina et al. [18] found that H₂O₂ at low concentrations increases the membrane AChE activity from erythrocytes; while H₂O₂ at high concentrations inhibits AChE activity. Such variation of AChE activity was explained by membrane modifications promoted by H₂O₂ as lipid peroxidation. Despite we found in our model lipid peroxidation induced by H₂O₂ [31], we
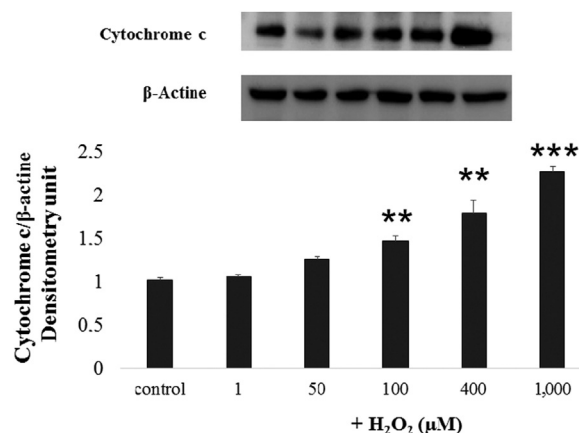
Fig. 6. Immunoblots of cytosolic cytochrome c using SH-SY5Y cells extracts as protein source, prepared after 24 h treatment with H₂O₂. Control condition (C) is referred to untreated cells. The results were normalized against beta actin. The extracts were obtained from three different cultures, each one performed by duplicated. **P < 0.01; ***P < 0.001.

discarded this mechanism considering data from the experiment with control cell extracts. In this control cells there is not lipid peroxidation, but H₂O₂ treatment increased AChE activity. Hence, lipid peroxidation could potentiate the effect of H₂O₂ on AChE activity but it is not the main cause in our case.

The increase in AChE activity observed in SH-SY5Y cells with H₂O₂ treatments seems to be mediated by a direct effect on the structure of the enzyme and not on the enzyme synthesis. This mechanism was assessed in the light of the following findings in this study: a) the kinetic parameters studied displayed a change from sigmoidal to hyperbolic behavior, impacting on the Vmax and not on the Km; b) the direct effect of H₂O₂ on AChE of control cell extracts showed a sharp increase of activity, which was dose- and time- dependent; and c) AChE levels were much lower in the presence of higher H₂O₂ concentrations, rather than increasing, as was expected in view of the increased activity of this enzyme. These results disagree with those of Zhang et al. [17] which found that during the apoptosis induced by H₂O₂ in 293 T cells there was increase in AChE expression via transcriptional activation of c-Jun N-terminal kinase (JNK). This contradictory effect could be due to a different response to H₂O₂ in the different cell lines 293 T (kidney human cells) and SH-SY5Y (neuroblastoma human cells).

The activator effect of H₂O₂ on control cell extracts was found to be dose-dependent, but no such effect was observed in cells treated for 24 h, where AChE activity was high at H₂O₂ concentrations of 1 μM and kept high until 1000 μM without a dose-dependent increase. This could be because the total AChE content decreased due to H₂O₂ treatment, and the enzyme activity was expressed depending on total protein content; hence, the ratio total proteins respect to AChE protein was modified by the treatment. However, in the experiment with control cell extract AChE protein level is the same in all H₂O₂ treatments, which means that total proteins/AChE protein ratio was unchanged.

In the case of the kinetic parameters, the sigmoidal behavior of control cells suggests that AChE of SH-SY5Y cell line is an allosteric enzyme. Furthermore, the positive value of the Hill coefficient is indicative that the substrate (ACh) presents positive cooperativity. Moreover, the value of the Hill coefficient can be related to the number of active sites of the enzyme, which in this case would agree with the subunit number found. Our control cells showed a Hill coefficient between 2 and 3, which could result from the mixture of monomers, dimmers and tetramers characteristic of this protein. On the other hand, the change observed in enzymatic behavior (Fig. 2c) suggests that H₂O₂ acts as an allosteric activator of AChE in SH-SY5Y cells. Furthermore, H₂O₂ would seem to act as a V-type heterotrophic effector, since it modified Vmax but not the Km. This behavior could be mediated by

oxidation of some amino acid from the active site. In this sense, Schallreuter et al. [16] observed that at concentrations lower than 1000 μM, H_2O_2 caused the activation of AChE via oxidation of methionine, cysteine and tryptophan. Other possibility could be the different AChE isoform composition induced by H_2O_2 in SH-SY5Y cells. In this sense, Bond et al. [29] treating astroglia cells with tert-butyl hydroperoxide, an oxidative stress generator, found that the increase of AChE activity promoted by this agent was via switching of mRNA between AChE-T and AChE-R isoforms.

4.2. H_2O_2 changed the AChE levels

Very often the studies conducted to ascertain the alterations of AChE are based on enzymatic activity determinations. Conversely, some authors highlight the importance of measuring AChE levels too, since the two parameters (activity and levels) do not always vary in the same direction [1].

The AChE bands detected in SH-SY5Y cells agree with those found by Darreh-Shori et al. [11] in the cortex and cerebrospinal fluid of AD patients, which could be explained because SH-SY5Y cells are nervous cells as has been mentioned above. In the present paper it has been quantified those bands which displayed changes after H_2O_2 treatment. The role of this isoform diversity is not well understood. The tetramer form (more than 200-kDa) was not detected in the present work perhaps because, as noted, protein content was measured under reduced conditions. The 132- and 100-kDa bands were considered dimer-trimer, and the smaller forms 70-, 65-, 55- and 50-kDa, monomers. Finally, the 40-kDa band could be defined as a proteolysis product of the enzyme as suggested by Berson et al. [8].

The PCA test showed a strong correlation between 132-, 70- and 50-kDa bands, quite close to the kinetic parameters (Vmax and Km) (Fig. 5a), suggesting that these three isoforms are more related to the catalytic activity. Moreover, Darreh-Shori et al. [11] report that 50-kDa isoform is included in heavy complexes because it dramatically increases when the experiment is conducted under reduced conditions. On the other hand, PCA test kept apart the 100-, 65- and 55-kDa isoforms, probably because they are also related to other functions (e.g. apoptosis and synaptogenesis, among others). Nonetheless, it is likely that there is also catalytic activity in the other three isoforms, since in our experiments 1000 μM of H_2O_2 kept the activity high while the bands of 132- and 70-kDa disappeared. H_2O_2 treatment modified AChE isoform profile from SH-SY5Y cell line. Although the consequence of this change is not well-known, AChE composition inside the cells could be important as depends on the physiological needs [32].

The treatment with increasing H_2O_2 concentrations gradually reduced the total AChE contents. The fall in AChE levels due to the treatment could mean that H_2O_2: a) caused degradation of the enzyme; b) induced AChE release; c) reduced the available amount of Ach and/ or cellular death by apoptosis.

Degradation of the enzyme as an isolated event did not seem the most probable mechanism, since the 40-kDa band was not significantly affected as compared to the control (Fig. 4c). AChE release has been reported by Hicks et al. [10], although the mechanism for this release is not well understood. Alternatively, AChE decrease due to cellular death is other possibility. In fact, our group [31] observed that a 24 h treatment with 400 μM of H_2O_2 in SH-SY5Y promote cell death by apoptosis and necrosis. Furthermore, we have found in the present study that H_2O_2 promoted significant increase of cytochrome c in the cytosol from 100 μM to 1000 μM.

Regarding the last possibility, the amount of Ach could decrease due to increased degradation, lower synthesis, or cell death.

4.3. H_2O_2 modified AChE isoform profile inducing apoptosis

The involvement of AChE in apoptosis has been fairly well established [33]. Actually, we found a positive correlations between the cytochrome c and the AChE 100- and 55-kDa isoforms (percentage value) ($P < 0.0001$; $P = 0.002$; respectively). The implication of 55-kDa isoform of AChE in apoptosis has been suggested by Xie et al. [34]. The possibility that H_2O_2 selectively affects cholinergic neurons, inducing their death by apoptosis and promoting cholinergic system dysfunction must be evaluated in future experiments.

In the present experiments, considerable changes were observed in all parameters measured following an insult of H_2O_2 1000 μM. Future studies would do well to assess the chronic effect of oxidative stress on AChE in animal models of neurodegenerative diseases.

5. Conclusions

In conclusion, present results suggest that: 1) oxidative stress promotes major disturbances in the levels of AChE, increasing its activity but oppositely reducing its levels, which could have important consequence in cholinergic transmission in SH-SY5Y cells (**Fig. 7; graphical abstract**); 2) H_2O_2 promotes cellular death by apoptosis, which together with AChE alterations would imply an additional mechanism in the hazardous effect of oxidative stress in this cell line. All these results demonstrate for the first time the possible mechanism by which an oxidant agent activate AChE in human neuroblastoma cells, and makes aware of the importance of studying the impact of antioxidant agents treatment as a suitable strategy to decrease ROS levels and consequently to block AChE activity and isoform profile alterations.

Conflict of interest

The authors declare that they are not conflicts of interest.

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