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Curcumin Protects SK-N-MC Cells from H_2O_2-Induced Cell Death by Modulation of Notch Signaling Pathway

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Abstract

Oxidative stress has been implicated to play a crucial role in the pathogenesis of Alzheimer's disease (AD). Currently, it is known that numerous signaling pathways involved in neurodegenerative disorders are activated in response to oxidative stress. Recent directions on AD treatments have focused on the use of antioxidants including Curcumin, a hydrophobic polyphenol derived from the rhizome of the herb *Curcuma longa*, to augment the intracellular antioxidant defences. In the present study, hydrogen peroxide (H_2O_2) was used to evaluate the effects of oxidative stress on apoptotic SK-N-MC cells death with focus on changes in activity of Notch signaling pathway. The extent of lipid peroxidation, protein oxidation and intracellular ROS (Reactive Oxygen Species) levels was investigated as oxidative stress biomarkers. Here, we showed that H_2O_2 reduced GSH levels and activity of antioxidant enzymes and also influenced Notch signaling activation. The present data concluded that Curcumin protected cells against oxidative stress-induced apoptosis.

Keywords

Oxidative Stress, Alzheimer's Disease (AD), Curcumin, Notch Signaling, Reactive Oxygen Species (ROS)

1. Introduction

Reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radicals ($^{\cdot}OH$) are produced during normal intracellular metabolism, e.g. mitochondrial metabolism [1] [2]. Oxidative stress arises due to disturbed equilibrium between pro-oxidant/antioxidant homeostasis

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that further takes part in generation of ROS and free radicals which are potentially toxic for neuronal cells [3]. Hydrogen peroxide (H_2O_2), one of the main reactive oxygen species, is produced during the redox process and is known to cause lipids peroxidation, proteins oxidation and DNA injuries with subsequent cellular apoptosis in various cell types [4] [5]. This has led to evaluating the impact of ROS on intracellular signaling cascades [6]. Notch receptors transmit signals between adjacent cells which are critical at multiple stages of development. These receptors are subjected to a series of proteolytic processing events by the presenilin-dependent γ-secretase that releases the Notch intracellular domain (NICD) [7]. NICD enters the nucleus and promotes transcription of target genes [8]. It has been demonstrated that Notch1 activity selectively influences apoptotic cell death in early neural progenitor cells as well as a p53-dependent pathway is activated in these cells in association with the extensive apoptosis induced by Notch activation [9]. Taken together, these studies suggest a role of Notch on apoptosis. One key regulator of Notch signaling is Numb, an evolutionarily conserved adapter protein discovered originally as an intrinsic cell fate determinant in *Drosophila* [10].

Previous findings support the idea that oxidative stress might indeed impair memory function. It would therefore seem that antioxidant molecules should be beneficial for treating Alzheimer's disease (AD). Naturally occurring phenolic compounds possess antioxidant properties which combat neurodegenerative diseases like AD [11]. Curcumin (diferuloylmethane), a hydrophobic polyphenol derived from the rhizome of the herb *Curcuma longa*, has been found to possess many interesting pharmacological and physiological activities [12], such as antioxidant, anti-inflammatory [13] [14], antimicrobial, and anticarcinogenic activities [15] [16]. Additionally, it has shown to be bothhepato- and nephro-protective [17] [18], thrombosis suppressing [19], myocardial infarction protective [20]. Recent evidence indicated that curcumin is a potent natural product to treat AD [21] [22] by scavenging free radicals [23] [24] and suppressing Aβ aggregation [25] [26]. The aim of the present study was to examine the effect of H_2O_2-induced oxidative stress on Notch signaling pathway and also the role of curcumin as a therapeutic agent on preventing or delaying ROS-induced apoptosis through its influence on this pathway.

2. Materials and Methods

2.1. Materials

The cell culture medium (RPMI-1640), fetal bovine serum (FBS) and penicillin–streptomycin were purchased from Gibco BRL (Life technology, Paisley, Scotland). Hydrogen peroxide (H_2O_2) and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). The SK-N-MC cell line was obtained from Pasteur Institute of Iran (Tehran, Iran). Curcumin, MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], phenylmethylsulphonyl fluoride(PMSF), leupeptin, pepstatin, aprotinin and dithionitrobenzoic acid (DTNB), GSH were purchased from Sigma Chem. Co. (Germany). 2',7'-Dichlorofluorescein diacetate ($DCFH_2$-DA) from Molecular Probe (Eugene, OR, USA). Ethidium bromide (EtBr) and acridine orange (Ao) were obtained from Pharmacia LKB Biotechnology AB Uppsala (Sweden). Anti-bcl-2, anti-Bax, anti-cleaved caspase-9, anti-p53, anti-p21 and anti-tubulin antibodies were purchased from Biosource (Nivelles, Belgium). Anti-NICD was obtained from cell signaling (MA, USA). Anti-HES1, anti-MDM2 and anti-Numb were purchased from Santa Cruz Biotechnology (Germany). Chemiluminescence detection system was purchased from Amersham-Pharmacia (Piscataway, NJ, USA).

2.2. Cell Culture

Human SK-N-MC cell line was cultured in RPMI-1640 medium supplemented with FBS (10%, v/v), streptomycin (100 µg/ml) and penicillin (100 µ/ml). The cells were incubated in a 5% CO_2 humidified atmosphere at 37˚C.

2.3. Cell Viability Determination

Cell viability was evaluated using the MTT assay. This method is dependent on the reduction of MTT to formazan by mitochondrial succinate dehydrogenase of the viable cells. The cells were seeded in 96-well plates at a concentration of 5×10^4/well for 24 h, and then pretreated with different concentrations of curcumin. Two hours later, H_2O_2 (150 µM) was added to the plate and incubated at 37˚C. After 24 h, the medium was discarded and 10 µl MTT was added to each well from the MTT stock solution (5 mg/ml). After 4 h of incubation, the supernatants were removed. The formazan crystals in each well were dissolved in 100 µl of DMSO, and the absor-

bance was measured via ELISA reader (Exert 96, Asys Hitch, Ec Austria) at a wavelength of 570 nm.

2.4. Morphological Study of the Cells

The cells (1×10^5 cells/well) were seeded in 12-well plates. They were pretreated with curcumin (0.1 µM) and then were treated with H_2O_2 (150 µM) for 24 h. Apoptosis was determined morphologically after staining the cells with acridine orange/ethidium bromide followed by fluorescence microscopy inspection. Cells were washed with cold PBS and adjusted to a cell density of 1×10^4 cells/ml of phosphate solution (1:1 v/v) then acridine orange/ethidium bromide solution (1:1 v/v) was added to the cell suspension in a final concentration of 100 µg/ml. The cellular morphology was evaluated by an Axoscope2 plus fluorescence microscope from Zeiss (Germany). All experiments were repeated three times and the number of stained cells was counted in 10 randomly selected fields. The extent of apoptosis was expressed as a percentage of the total cell count.

2.5. DCF Method for Detection of Intracellular ROS

ROS levels were measured by flow cytometry using 2',7'-dichlorodihydro-fluorescein diacetate ($DCFH_2$-DA), which is converted into a non-fluorescent derivative ($DCFH_2$) by intracellular esterases. The oxidation of $DCFH_2$ by intracellular ROS mainly H_2O_2, results in fluorescent DCF which stains the cells [27]. Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells [28]. In this experiment, cells were pretreated with curcumin (0.1 µM). After 2 h, H_2O_2 (150 µM) was added to the cells. Then, cell samples were incubated in the presence of 10 µM DCFH2-DA in phosphate buffered saline (PBS) at 37˚C for 30 min then washed two times with PBS to remove the extracellular $DCFH_2$-DA and the fluorescence intensity was evaluated using flow cytometery technique (Partec PAS, Germany).

2.6. Antioxidant Assays

2.6.1. Catalase Activity Assay

CAT activity was measured by the method of Aebi, 1984 [29]. To determine enzyme activity, 200 µl of each cell lysate was added to a cuvette containing 1.995 mL of 50 mM phosphate buffer (pH 7.0). Reaction was started by addition of 1.0 mL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm. Activity of CAT was expressed as $\times 10^{-1}$ k·mg^{-1} protein, where k represents the rate constant of the first order reaction of CAT. Protein concentration was determined by the method of Lowry [30].

2.6.2. Glutathione Peroxidase Activity Assay

Glutathione peroxidase activity was assayed in a 1mL cuvette containing 0.890 mL of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U/ml GSH reductase and 1 mM GSH. Cell lysate was added to make a total volume of 0.9 ml. The reaction was initiated by the addition of 100 µL of 2.5 mM H_2O_2, and the conversion of NADPH to $NADP^+$ was monitored with a spectrophotometer at 340 nm for 3 min. GPx activity was expressed as nmoles of NADPH oxidized to $NADP^+$/min/mg protein, using a molar extinction coefficient of 6.22×10^6 ($cm^{-1} \cdot M^{-1}$) for NADPH [31].

2.7. Determination of Lipid Peroxidation

To determine lipid peroxidation, the level of thiobarbituric acid reactive substance (TBARS), mainly malonydialdehyde (MDA), was assayed spectrophotometrically. The extent of MDA in the presence and absence of H_2O_2 was evaluated based on the amount of TBARS according to the double heating method [32]. MDA measurement was based on absorbance of the purple malondialdehyde (MDA)-TBA complex at 532 nm. Briefly, the cells cultured in 12-well plates were pretreated with curcumin (0.1 µM). After drug treatment, cells were exposed to 150 µM H_2O_2 for 24 h. Then, the cell lysates were mixed with 0.5 mL of 10% trichloroacetic acid and heated at 95˚C for 15 min. After cooling to room tempreture, the samples were centrifuged at 3000 rpm for 10 min and 2 ml of each sample supernatant was mixed with TBA solution (0.67% w/v). Each tube was then placed in boiling water bath for 15 min. After cooling to room temperature, the absorbance was read at 532 nm with respect to the blank solution. The concentration of MDA was calculated based on the extinction coefficient of the TBA-MDA complex ($\varepsilon = 1.56 \times 105$ $cm^{-1} \cdot M^{-1}$) and it was expressed as nmol/mg of protein.

2.8. Determination of Protein Oxidation

Widely used methods for determination of protein carbonyl (PCO) content, as a marker of oxidative stress, utilize the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form protein-bound 2,4-dinitrophenylhydrazones. For determination of PCO, 1 ml of 10 mM DNPH in 2 M HCl was added to cell lysates. Samples were incubated for 1 h at room temperature. Then, 1 ml of trichloroacetic acid (TCA 10% w/v) was added to the mixture and centrifuged at 3000 g for 10 min. The protein pellets were washed three times with 2 ml of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 ml of guanidine hydrochloride (6 M, pH 2.3). The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($\varepsilon = 2.2 \times 10^4$ $cm^{-1} \cdot M^{-1}$) and was expressed as nmol/mg protein.

2.9. Intracellular Glutathione Evaluation

Intracellular glutathione level was determined using dithionitrobenzoic acid (DTNB) method. In the present study, cells were pretreated with curcumin (0.1 and 0.2 µM). Two hours later, H_2O_2 (150 µM) was added to the cells followed by incubation at 37˚C for an additional 24 h. The concentration of GSH was determined spectrophotometrically at 412 nm in the whole cell lysate using DTNB and the reduced glutathione was expressed as µg/mg of protein [33].

2.10. Immunoblot Analysis

After pretreatment of SK-N-MC cells with curcumin (0.1 µM), H_2O_2 (150 µM) was added to the cells and incubated at 37˚C for 24 h. The cells were harvested and lysed using lysis buffer containing 1% Triton X-100, 1% SDS, 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM sodium pyrophosphate, 2 mM Na_3VO_4, 1 mM NaF, 0.5% sodium deoxycholate, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 µg/ml leupeptin, 1 µg/ml pepstatin and 60 µg/ml aprotinin. Protein concentration of each sample was determined using Lowry's method. Equal quantities of protein (50 µg) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electrically transferred to nitrocellulose membranes. Transfer of proteins was assessed by ponceau-red staining. The filter membranes were blocked in Tris-buffered saline pH 7.4 containing 0.1% Tween-20 and 5% BSA (bovine serum albumin) overnight at 4˚C. The blocked blots were incubated with primary antibodies for 1 h at room temperature using antibody dilutions as recommended by the manufacturer in Tris-buffered saline pH 7.4, 0.1% Tween-20 Following 1 h incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Biosource, Belgium), the proteins were detected by an enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia, Piscataway, NJ) according to the manufacturer's instructions.

2.11. Statistical Analysis

Data were expressed as percent of values in untreated control cells and each value represents the mean ± S.D. (n = 3). The significant differences between the means of the treated and untreated groups were calculated by unpaired Student's t-test and p-values less than 0.05 were considered significant.

3. Results

3.1. Curcumin Improves the Viability of H_2O_2-Treated Cells

To find the concentration of H_2O_2 where 50% of cells are viable, SK-N-MC cells were treated with a range of concentrations of H_2O_2 between 0 and 400 µM followed by further incubation for 24 h. At the end of incubation period, cell viability was established by the MTT assay. Based on **Figure 1(a)**, exposure of the cells to H_2O_2 has caused reduction in viability by almost 18.1%, 25.8%, 52.9%, 73.3% and 81.9% at concentrations of 50, 100, 150, 300 and 400 µM, respectively. Regarding these data, the rest of experiments have been carried out at a H_2O_2 concentration of 150 µM. Pretreatment of the cells with curcumin for 2 h (0.1 - 0.5 µM) and subsequent treatment with 150 µM H_2O_2 for 24 h enhanced the extent of viability in a concentration-dependent manner. As it is evident from **Figure 1(b)**, curcumin at concentrations of 0.1, 0.2 and 0.5 µM restored the cell viability by 25.9%, 15.9% and 7.0%, respectively. However, higher concentrations of curcumin were cytotoxic. Thus, 0.1 µM was selected as the best effective dose of curcumin with maximum antioxidant activity.

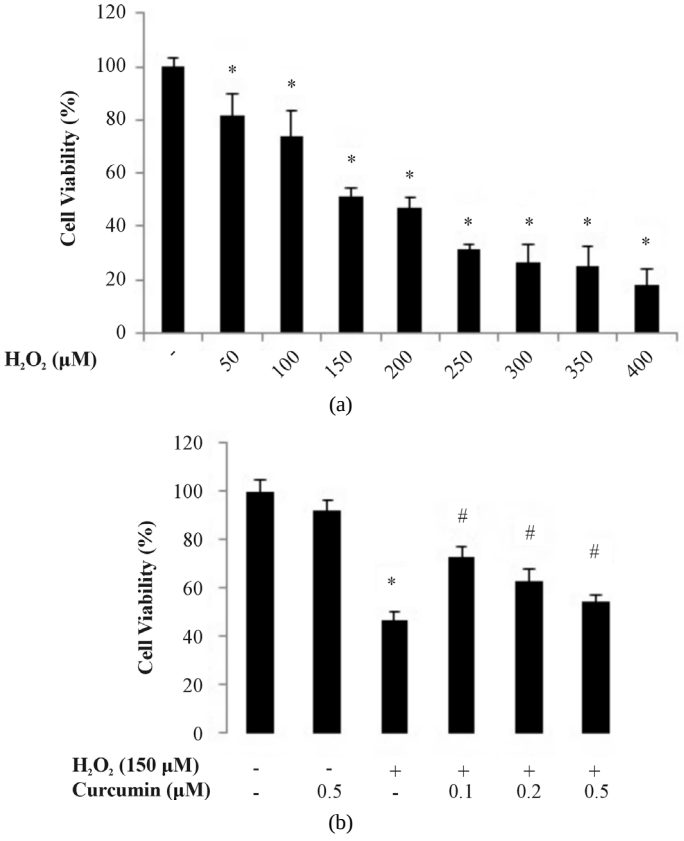

Figure 1. Effect of H_2O_2 on SK-N-MC cells viability. SK-N-MC cells were treated with different concentrations of H_2O_2 (0 - 400 μM) for 24 h (a). Protective effects of of curcumin (0 - 0.5 μM) on H_2O_2-induced cell death estimated with MTT assay (b). The data are the means of three independent measurements ± SD ($P < 0.05$). *Significantly different from control cells ($P < 0.05$); #Significantly different from H_2O_2-treated cells ($P < 0.05$).

3.2. Morphological Study of the Cells

Cell death in H_2O_2-treated cells was evident. This is in contrast with the cells pre-treated with curcumin followed by exposure to H_2O_2 which showed more homogeneity in shape and size, as well as detachments from culture plates were significantly less.

The quantitative analysis of apoptosis was evaluated by acridine orange/ethidium bromide (Ao/EtBr) double staining, using a fluorescence microscope (**Figure 2(a)** and **Figure 2(b)**. Pre-treatment of the cells with curcumin (0.1 μM) decreased the extent of cell apoptosis to 27.7% from 45.5% observed among the cells exposed solely to H_2O_2. Uniform bright green nuclei are representative of viable cells. However, apoptotic cells show bright green and orange area of condensed or fragmented chromatin in their nuclei. The necrotic cells reveal uniform bright orange nuclei.

3.3. Curcumin Attenuates H_2O_2-Induced ROS Generation

To examine whether curcumin could affect intracellular ROS production induced by H_2O_2, we measured intracellular ROS levels using $DCFH_2$-DA staining. H_2O_2 (150 μM) significantly enhanced ROS production, while pretreatment of the SK-N-MC cells with 0.1 μM curcumin attenuated H_2O_2-induced ROS generation (**Figure 3**).

3.4. Restoration of Catalase and Glutathione Peroxidase Activities by Curcumin

To study whether the effect of curcumin is related to the alteration of intracellular antioxidant status, the activi-

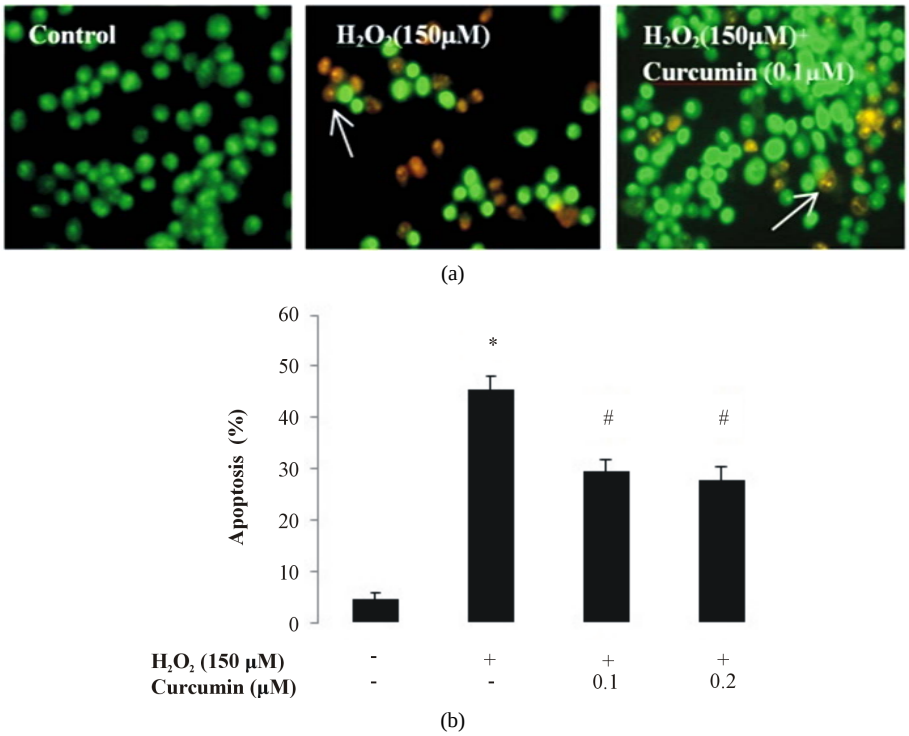

Figure 2. Morphological evaluation of cells exposed to curcumin (0.1 μM) for 2 h followed by exposure to 150 μM H_2O_2 for 24 h. *White arrow* indicates late phase of apoptosis (a). Protective effects of curcumin on the extent of apoptosis (b). The data are the means of three independent measurements ± SD ($P < 0.05$). *Significantly different from control cells ($P < 0.05$); #Significantly different from H_2O_2-treated cells ($P < 0.05$).

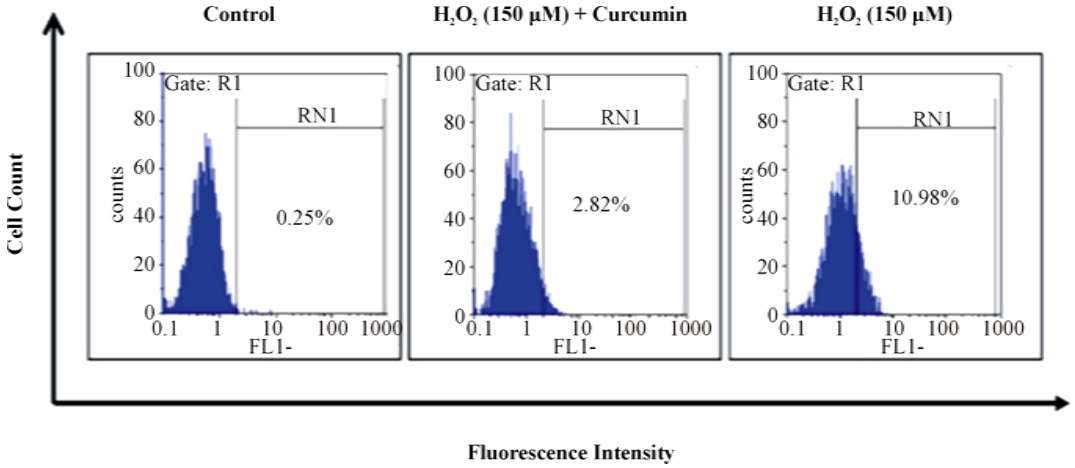

Figure 3. Inhibitory effects of curcumin (0.1 μM) on intracellular ROS production induced by H_2O_2. SK-N-MC cells were pretreated with curcumin (0.1 μM) for 2 h before treatment with 150 μM H_2O_2 for 24 h. Then, cells were incubated with 2',7'-dichlorofluorescein diacetate ($DCFH_2$-DA) (10 μM) and the fluorescence intensity of 10,000 cells was analyzed using a flow cytometer. The graph is a prototype of three independent experiments.

ties of glutathione peroxidase and catalase, the main enzymatic defenses against ROS, were determined at different concentrations of curcumin among the H_2O_2-treated cells. H_2O_2 (150 μM) reduced catalase activity to 0.98 k·mg^{-1} protein compared to control cells (2.5 k/mg protein). However, pre-treatment of the cells with curcumin at concentrations of 0.1 and 0.2 μM increased the catalase activity to 1.72 and 1.32 k/mg protein, respec-

tively (**Figure 4**). As shown in **Figure 4** glutathione peroxidase activity reduced among the H_2O_2-treated cells (1.14 nmol/min/mg protein) compared to untreated control cells (2.14 nmol/min/mg protein). Pre-treatment of the cells with curcumin at concentrations of 0.1 and 0.2 µM increased the glutathione peroxidase activity to 1.73 and 1.53 nmol/min/mg protein, respectively.

3.5. Curcumin Represses H_2O_2-Induced Lipid Peroxidation

It is known that H_2O_2-induced generation of ROS results in an attack on cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation. One of the final products of the lipid peroxidation process is malondialdehyde (MDA). As shown in **Table 1**, MDA level was significantly increased among the cells exposed to 150 µM H_2O_2 (2.25 nmol/mg protein). However, pretreatment with curcumin (0.1, 0.2 µM) decreased MDA content to 0.87 and 1.05 nmol/mg protein, respectively.

3.6. Curcumin Decreases H_2O_2-Induced PCO Formation

The concentration of carbonyl groups generated by oxidative stress is a good measure of ROS-mediated protein oxidation. After treatment with H_2O_2 (150 µM), the PCO formation increased to 3.24 nmol/mg protein in comparison with the control cells (0.49 nmol/mg protein). However, pretreatment with curcumin (0.1, 0.2 µM) reduced protein carbonyl formation to 1.29 and 1.9 nmol·mg^{-1} protein, respectively (**Table 1**).

3.7. Curcumin Prevents H_2O_2-Induced GSH Depletion

GSH has nonenzymatic antioxidant activity and it is present primarily in its reduced from (GSH) within the cells. It also regulates the activities of many enzymes with critical thiol groups, and protection against oxidation of protein–SH groups. The GSH level changes during aging, oxidative stress conditions and disease. Thus, it is considered as a biomarker of oxidative stress [34]. The intracellular concentration of GSH was determined to elucidate the mechanism underlying the antioxidant function of curcumin. Based on DTNB assay, the intracellular GSH concentration of SK-N-MC cells was reduced to 4.36 µg·mg^{-1} protein when the cells were treated with 150 µM H_2O_2 for 24 h (**Figure 5**). However, the cellular GSH contents of the cells pretreated with 0.1 and 0.2 µM curcumin were 7.9 and 6.2 µg/mg protein, respectively, relative to the control cells (9.38 µg/mg). In other words, the intracellular GSH contents have increased by 37.74% and 19.61% among the cells pretreated with 0.1 and 0.2 µM curcumin, respectively, relative to the H_2O_2-treated cells.

3.8. Curcumin Decreases Bax/Bcl2 Ratio and Procaspase-9 Activation in H_2O_2-Treated Cells

Previous findings suggest that H_2O_2-induced apoptosis correlates with the level of expression of apoptosis regulatory proteins, Bax and bcl-2 in cells. Bax is a pro-apoptotic member of the Bcl-2 family which undergoes conformational changes and may form a pore big enough for the apoptogenic proteins to pass through. Cytochrome c, a component of the mitochondrial electron transfer chain, initiates procaspase-9 activation and further activation of procaspase-3 and cell death through apoptosis when released from mitochondria during apoptosis. Pretreatment of cells with curcumin, prior to H_2O_2 treatment, reduced procaspase-9 activation (**Figure 6**). Our data suggested that Bax/bcl-2 ratio, as an index of apoptosis, increased in response to H_2O_2. However, curcumin reduced this ratio.

Table 1. Effects of different concentration of curcumin on protein carbonyl (PCO) formation and lipid peroxidation in H_2O_2-treated cells. The data are the means of three independent measurements ± SD ($P < 0.05$). *Significantly different from control cells ($P < 0.05$); $^{\#}$Significantly different From H_2O_2-treated cells ($P < 0.05$).

Curcumin (µM)	H_2O_2 (150 µM)	MDA (nmol·mg^{-1})	PCO (nmol·mg^{-1})
0	−	0.57 ± 0.05	0.49 ± 0.05
0	+	2.25 ± 0.02*	3.24 ± 0.20*
0.1	+	0.87 ± 0.03$^{\#}$	1.29 ± 0.04$^{\#}$
0.2	+	1.05 ± 0.03$^{\#}$	1.9 ± 0.07$^{\#}$

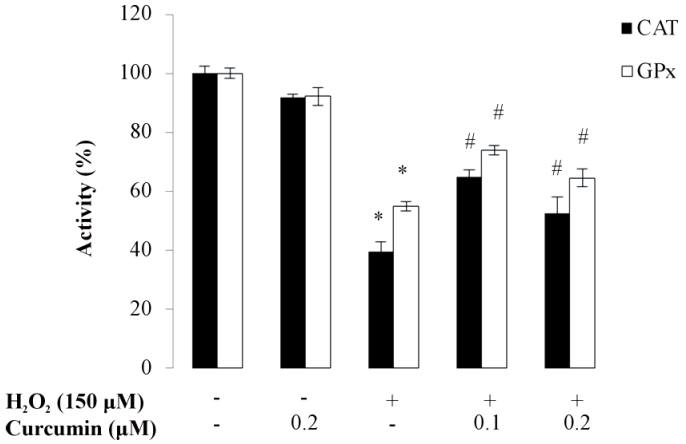

Figure 4. Protective effects of different concentrations of curcumin on catalase/glutathione peroxidase activities. SK-N-MC cells were pretreated with curcumin (0.1, 0.2 µM) for 2 h. Then, cells exposed to H_2O_2 (150 µM). The data are the means of three independent measurements ± SD ($P < 0.05$). *Significantly different from control cells ($P < 0.05$); #Significantly different from H_2O_2-treated cells ($P < 0.05$).

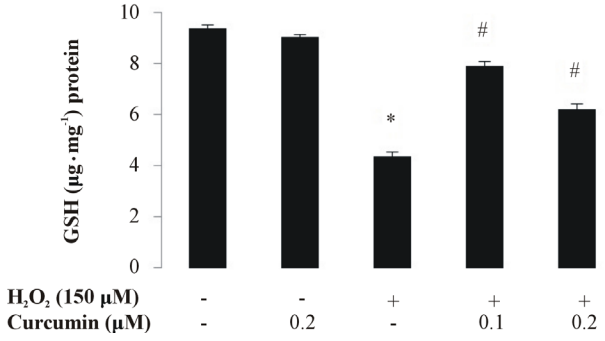

Figure 5. Effects of curcumin on intracellular reduced glutathione (GSH) levels in SK-N-MC cells. The cells were pretreated with curcumin (0.1, 0.2 µM) and then exposed to H_2O_2 (150 µM) for 24 h. Intracellular glutathione evaluated with DTNB. The data are the means of three independent measurements ± SD ($P < 0.05$). *Significantly different from control cells ($P < 0.05$); #Significantly different from H_2O_2-treated cells ($P < 0.05$).

3.9. Effect of Curcumin on H_2O_2-Induced Expression of P53

P53 is one of the major tumour suppressor proteins. MDM2 protein binds to p53 and hinders its transcriptional activity. In addition, MDM2 regulates p53 half-life through its E3 ubiquitin-ligase activity [35]. To evaluate the p53 downstream genes expressed following H_2O_2 insult, we focused on the expression of cyclin-dependent kinase inhibitor p21 [36]. In order to discover the effects of curcumin on p53 expression and cell survival, the cells were pretreated with curcumin for 2 h followed by H_2O_2 treatment. Treatment with H_2O_2 resulted in upregulation of p53 and p21 expression which is associated with the downregulation of MDM2. In contrast, curcumin reversed the effects of H_2O_2 on p53, p21 and MDM2 levels (**Figure 7**).

3.10. Effect of Curcumin on H_2O_2-Induced Expression of NICD, HES1 and NUMB

Numb was originally discovered as an intrinsic cell fate determinant in Drosophila by antagonizing Notch signaling activity. Therefore, we hypothesized that the effect of Numb on H_2O_2-induced apoptosis might be mediated by Notch signaling. To address this issue, we first examined the effect of H_2O_2 on the expression of the

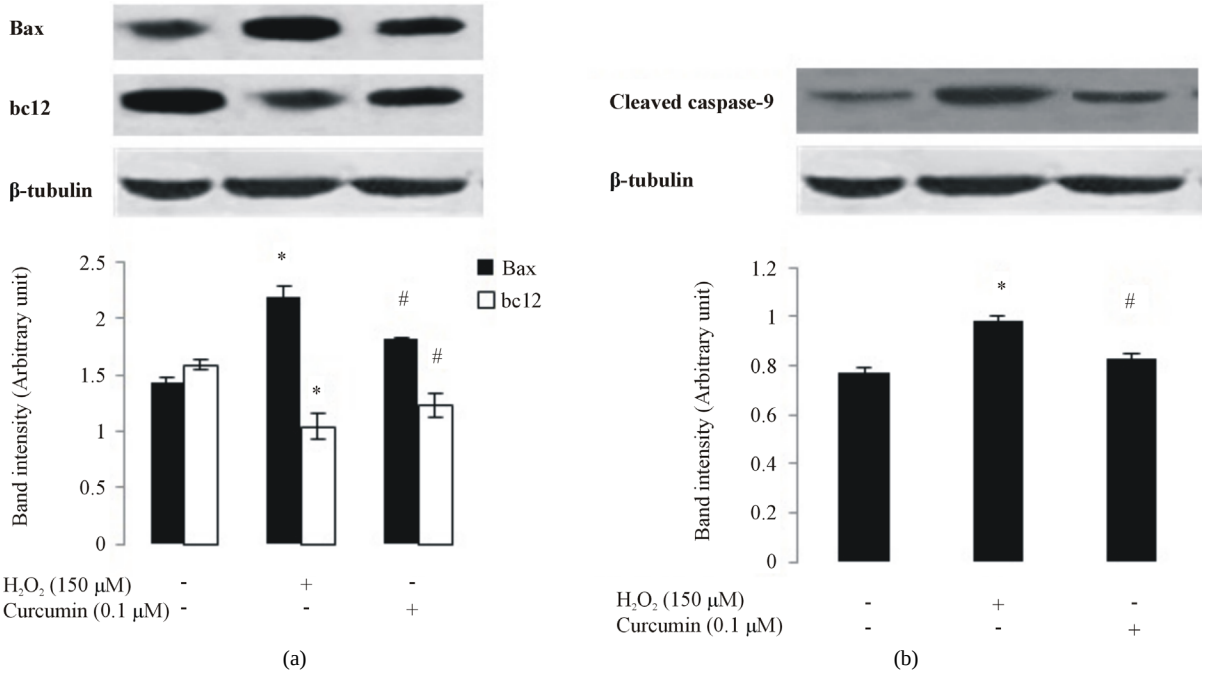

Figure 6. Analyses of Bax, bcl2 levels and procaspase-9 activation in H_2O_2-treated SK-N-MC cells. Cells were pretreated with 0.1 μM curcumin followed by incubation with 150 μM H_2O_2 for 24 h. Bax, bcl-2 (a) and cleaved caspase-9 (b) proteins expression in H_2O_2-treated SK-N-MC cells was measured by western blot analysis using monoclonal antibodies against each factor. Intensity of each band was estimated by densitometric analysis. All data are representative of three independent experiments ± SD ($P < 0.05$). *Significantly different from control cells ($P < 0.05$); #Significantly different From H_2O_2-treated cells ($P < 0.05$).

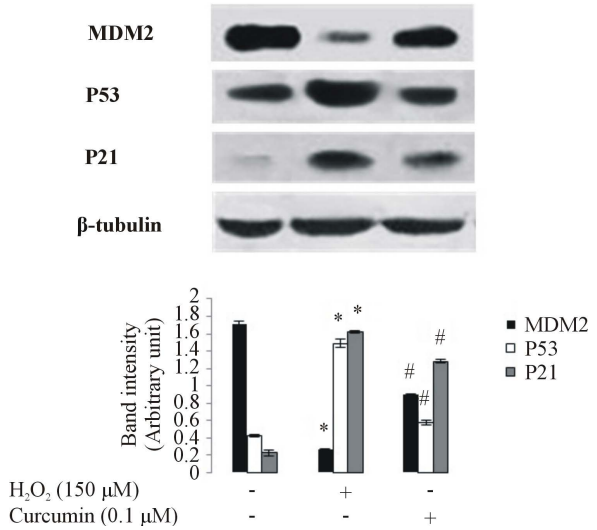

Figure 7. Analyses of MDM2, p53 and p21 expression levels in H_2O_2-treated SK-N-MC cells. Cells were pretreated with 0.1 μM curcumin followed by incubation with 150 μM H_2O_2 for 24 h. MDM2, p53 and p21 proteins expression in H_2O_2-treated SK-N-MC cells was analysed by western blot analysis using monoclonal antibodies against each factor. Intensity of each band was estimated by densitometric analysis. All data are representative of three independent experiments ± SD ($P < 0.05$). *Significantly different from control cells ($P < 0.05$); #Significantly different From H_2O_2-treated cells ($P < 0.05$).

Notch intracellular domain (NICD) and HES1, the downstream target gene of Notch signaling. As shown in **Figure 8**, H_2O_2 treatment significantly increased the NICD and HES1 expression, suggesting that H_2O_2 could activate Notch signaling. On the other hand, H_2O_2 treatment resulted in down-regulation of numb expression in a time-dependent manner as well as curcumin decreased NICD and HES1 levels. Taken together, these data provided evidence that curcumin could protect SK-N-MC cells from H_2O_2-induced apoptosis by Numb up-regulation which antagonize Notch signaling activity.

4. Discussion

The AD brain is characterized by extensive oxidative stress [37] [38]. Currently, it is known that numerous signaling pathways which are involved in neurodegenerative disorders including AD are activated in response to oxidative stress. Thus, removal of excess reactive oxygen species or suppression of their generation may be effective in preventing oxidative cell death. Regarding the previous findings, future directions on AD treatments will focus on the use of antioxidants to contribute to the neuroprotection and potential enhancement of the intracellular antioxidant mechanisms.

Curcumin with its proven antioxidant properties has been shown to have several therapeutic effects [39]

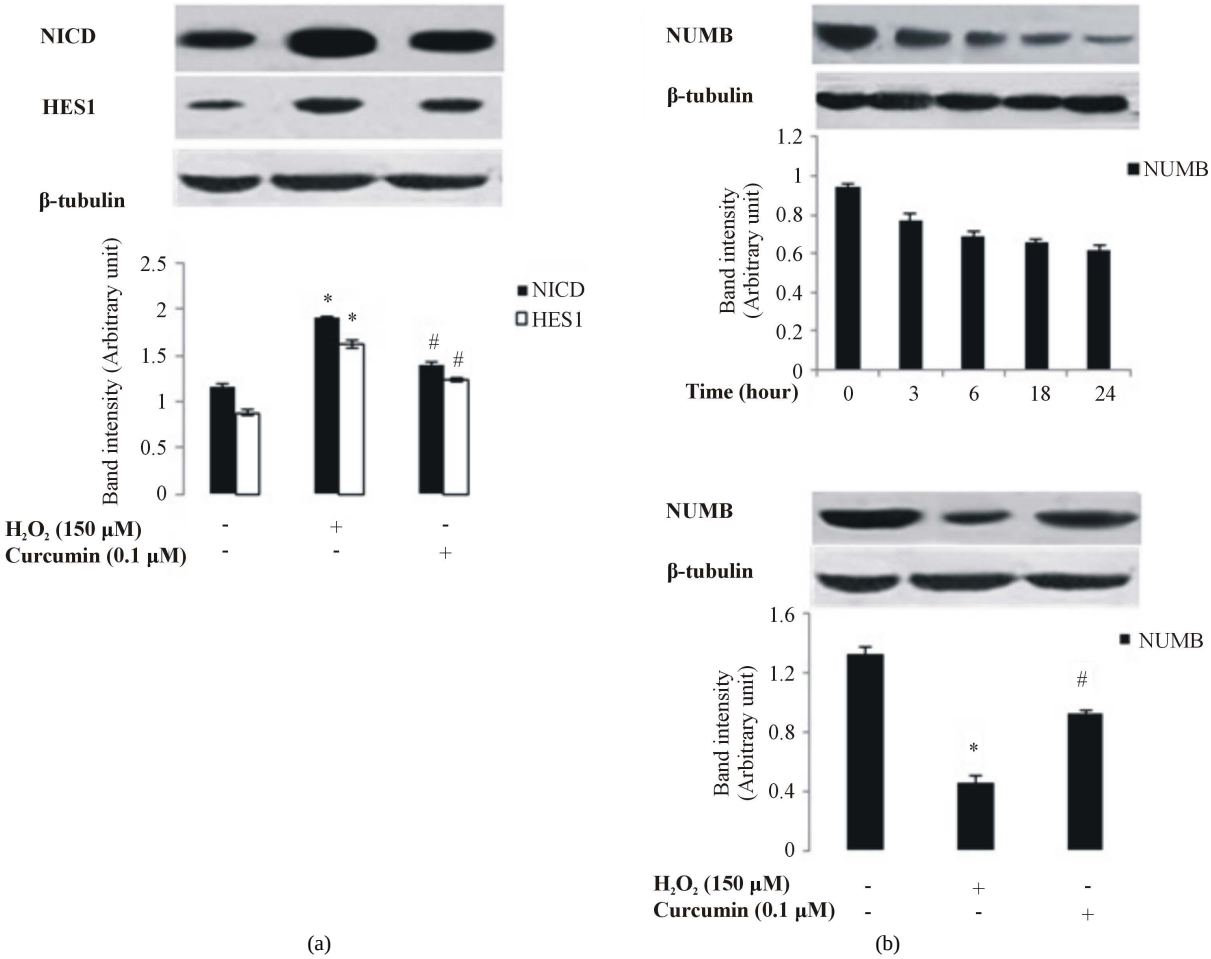

Figure 8. Analyses of NICD, HES1 and Numb expression levels in H_2O_2-treated SK-N-MC cells. Cells were pretreated with 0.1 μM curcumin followed by incubation with 150 μM H_2O_2 for 24 h. NICD and HES1 proteins expression in H_2O_2-treated SK-N-MC cells was measured by western blot analysis using monoclonal antibodies against each factor. The intensity of each band was estimated by densitometric analysis. (a). Cells were pretreated with 0.1 μM curcumin followed by incubation with 150 μM H_2O_2 for 24 h and Numb protein level in H_2O_2-treated SK-N-MC cells analysed by western blot (b). All data are representative of three independent experiments ± SD ($P < 0.05$). *Significantly different from control cells ($P < 0.05$); #Significantly different From H_2O_2-treated cells ($P < 0.05$).

which could act as a free radical scavenger by inhibiting lipid peroxidation and oxidative DNA damage [40]. The strong antioxidant activity of curcumin makes it an interesting candidate for use in counteracting oxidative stress-induced damage. Our results indicated that H_2O_2 induced a significant decrease in viability among the cells and increased the number of apoptotic cells, as confirmed by Bax/bcl-2 ratio and the cleaved caspase-9 level. However, pre-treatment of the cells with curcumin restored cell viability as well as catalase and glutathione peroxidase activities. Moreover, the protective effects of curcumin on the stressed cells decreased intracellular ROS levels, protein oxidation and lipid peroxidation. Protein oxidation could lead to aggregation or dimerization of proteins and subsequent accumulation of the oxidized proteins as cytoplasmic inclusions, such as tau aggregation in the form of tangles and amyloid-β aggregation as senile plaques, as observed in AD [41] [42]. On the other hand, 4, hydroxy-2-nonenal, main lipid peroxidation product, alters conformation and function of proteins [43] [44]. Taken together, curcumin offered significant neuroprotection through inhibition of lipid peroxidation, increase in endogenous antioxidant defense enzymes, decrease of ROS levels and reduction in protein carbonyl formation. Our data provide the evidence that curcumin has effective hydrogen peroxide scavenging activity. In the present study, we employed approaches to determine the effects of curcumin on regulation of Notch signaling pathway with pivotal roles in SK-N-MC cells death.

Notch signaling regulates cell proliferation in a cell type-specific manner. Its activation can increase proliferation in mammalian astrocytes [45]-[47]. However, Notch activation promotes cell cycle arrest in the vertebrate retina [48] [49]. Involvement of Notch signaling in ROS-mediated apoptosis has been shown in some studies. A previous study concluded that induction of extensive apoptotic cell death in a subset of neural progenitor cells via Notch activation promotes apoptotic cell death via a p53-dependent pathway [9]. In other words, Notch activation in neural progenitor cells leads to elevated levels of nuclear p53 and transcriptional upregulation of the target genes Bax, and the promotion of apoptotic cell death, demonstrating a crucial role for Notch in the regulation of apoptosis in early neural progenitors through a p53-dependent mechanism although the exact mechanism is not yet completely clear. [9]. These findings support that Notch may act as a specification marker for cell death under stress conditions.

To investigate the mechanism underlying oxidative stress induced apoptosis, we first investigated p53, p21 and MDM2 expression. The relatively high levels of p53 expressed in H_2O_2-treated cells suggest that p53 tumor suppressor protein is involved in H_2O_2-induced apoptosis. P21 protein is also upregulated in response to H_2O_2 which may be induced by either a p53-dependent or p53-independent mechanism [50]. We analyzed the Notch1 intracellular domain (NICD) and HES1 expression in response to H_2O_2 treatment and demonstrated that exposure of SK-N-MC cells to H_2O_2 increased NICD and HES1 expression. The fact that HES1 modulate p53 signaling by inhibiting MDM2 function and is regulated by Notch signaling supports a model for cell-type-specific crosstalk between the Notch and p53 signal transduction pathways in which Notch activates p53 by upregulating target genes such as Hes1. This finding provides an explanation for the apoptotic effect of Notch in some cell types [51].

It has been demonstrated that Numb promotes NICD ubiquitination and degradation [52] [53]. Our present data showed that exposure of SK-N-MC cells to H_2O_2 decreased Numb content in a time-dependent manner. However, curcumin, remarkably raised the Numb protein level leading to inhibition of Notch signaling activity in SK-N-MC cells exposed to H_2O_2. Based on the present data we hypothesized that Numb could act as a survival factor capable of regulating H_2O_2-induced apoptosis by modulating the activity of Notch signaling (**Figure 9**). Overall, curcumin could be an excellent option in synthesis of natural-based drugs for treatment of neurodegenerative disorders including AD.

5. Conclusion

In the present study, (H_2O_2) was used to evaluate the effects of oxidative stress on apoptotic SK-N-MC cells death with focus on changes in activity of Notch signaling pathway. Here, we showed that H_2O_2 reduced GSH levels and activity of antioxidant enzymes and also influenced Notch signaling activation. The present data concluded that curcumin protected cells against oxidative stress-induced apoptosis.

Conflict of Interests

The authors declare that they have no conflict of interests.

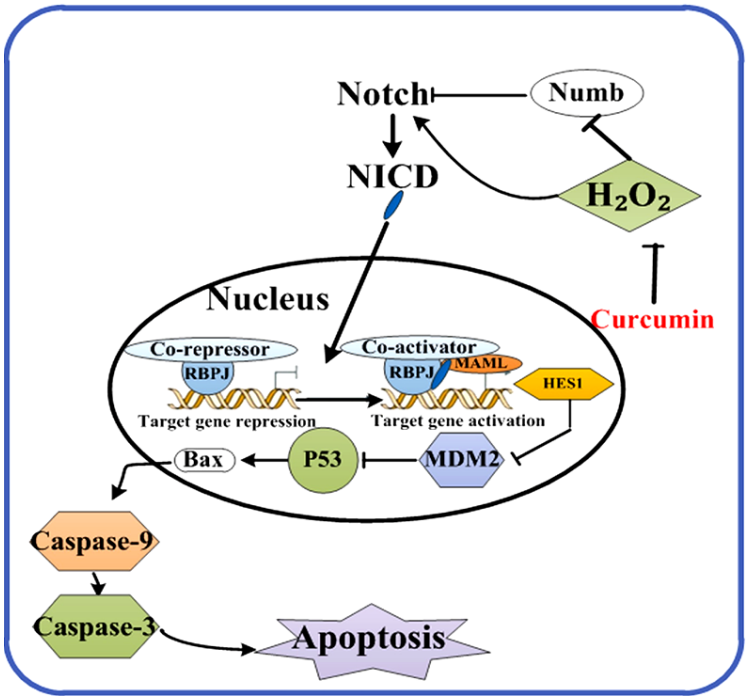

Figure 9. A schematic representation of signaling events in H_2O_2-induced SK-N-MC cell death. Arrows: H_2O_2-induced molecular events leading to cell death.

Acknowledgments

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Abbreviations

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
GSH, Glutathione;
DTNB, Dithionitrobenzoic acid;
MDA, Malondialdehyde;
PBS, Phosphate buffer saline;
PCO, Protein carbonyl.