Reactive Oxygen Species-Induced Apoptosis in PC12 Cells and Protective Effect of Bilobalide

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ABSTRACT
Although clinical studies have demonstrated that EGb 761, a standard extract of Ginkgo biloba, was effective in mild-to-moderate dementia of the Alzheimer's disease patients, the mechanism underlying its neuroprotective effect remains unclear. In this study, effects of bilobalide, the main constituent of the nonflavone fraction of EGB 761, on reactive oxygen species (ROS)-induced apoptosis in PC12 cells was studied. Exposure of cells to xanthine (100 μM)/xanthine oxidase (150 mU/ml) (ROS producer) resulted in a characteristic DNA fragmentation and an increase in the apoptosis rate. When p53, c-Myc, Bcl-2, Bcl-xL, and Bak were measured by flow cytometry and the activities of caspase-1- and caspase-3-like protease determined with Ac-YVAD-AMC or Ac-DEVD-AMC as substrates, the profile of ROS-induced changes in these apoptosis regulatory and effector proteins suggests that elevation of c-Myc, p53, and Bak and activation of caspase-3 play an important role in the apoptosis. When cells were treated with ROS and bilobalide (25–100 μM) simultaneously, a dose-dependent reduction in the apoptotic rate was found. The percentage of cells with positive staining for c-Myc and p53 decreased from 27.8 and 50.1% to 16.7 and 23.2%, respectively, when bilobalide (25 μM) was present. Bilobalide also reduced ROS-induced elevation of Bax and activation of caspase-3 effectively. Our results provide the first direct evidence that bilobalide can protect neurons against oxidative stress. Bilobalide may block the apoptosis in the early stage and then attenuate the elevation of c-Myc, p53, and Bak and activation of caspase-3 in cells.

Recently, placebo-controlled, double-blind, and randomized trials have demonstrated that EGb 761, a standard extract of Ginkgo biloba (EGb) was effective in mild-to-moderate dementia of the Alzheimer's disease patients (Le Bars et al., 1997; Maurer et al., 1997). Although pharmacological studies have shown that EGb improves cerebral blood flow in models of focal ischemia (Oberpichler et al., 1988; Kriegstein et al., 1995); exerts neuroprotective effect during ischemia (Smith et al., 1996); and attenuates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced nigrostriatal dopaminergic neurotoxicity in C57 mice (Wu and Zhu, 1999), the mechanism underlying its neuroprotective effect remains unclear. EGb is a complex mixture containing a number of flavonoids (e.g., kaempferol, quercetin, and isorhamnetin derivatives), terpenes (e.g., ginkgolides A, B, C, and J, and bilobalide), and other various constituents (DeFeudis, 1991). The early experiments of Oberpichler et al. (1988) showed that the nonflavone fraction, and not the flavonoid glycosides, protected brain tissue against hypoxic damage. Kriegstein et al. (1995) demonstrated that ginkgolides A and B and bilobalide reduced the infarct volume after focal ischemia in rodents and that ginkgolide B and bilobalide reduced the number of damaged neurons in culture after glutamate treatment or hypoxia. Recently, we demonstrated that bilobalide, the major constituent of G. biloba, could protect against β-amyloid toxicity (Zhou et al., 2000). Evidence also has shown that EGb and some of its constituents could inhibit serum deprivation- or staurosporine-induced apoptosis and that bilobalide was the most potent constituent (Ahlemeyer et al., 1999). However, the mechanism of neuroprotection by bilobalide is still unclear.

Apoptosis is an active process of cell destruction. A number of genes and their proteins can influence or determine the progression of apoptosis. The bcl-2 family, a group of apoptosis regulatory genes, has received particular attention. In this family, bcl-2 and bcl-xl are antiapoptotic, whereas bax, bcl-xS, bad, bak, and bik are proapoptotic. Dimerization of antiapoptotic factor with proapoptotic factor is assumed to be a critical interaction. For example, cells continue to survive if Bcl-2 predominates over Bax. In contrast, a higher concentration of Bax compared with Bcl-2 enhances cell susceptibility to apoptosis (Oltvai et al., 1993; Adams and Cory, 1998). p53, another important cell death regulatory factor, can act as a transcriptional activator of bax gene. The acti-
vation of p53 may lead to an increase in the Bax/Bcl-2 ratio and therefore contribute to the initiation of apoptosis (Miyashita and Reed, 1995; Hughes et al., 1997). Normally, c-Myc is required for cell cycle entry and promotes cell proliferation when growth factors are available. However, c-Myc induces apoptosis when growth factors are not available (Staunton and Gaffney, 1998). As to the intracellular death effectors, the most important family in the process of apoptosis is caspases, a group of cysteine aspartate-specific proteases (Gorman et al., 1998). Clarification of the effect of EGB and its components on the expression of these molecules involved in apoptosis may give us new insight into the mechanism of neuroprotection by bilobalide and other components of EGB.

Reactive oxygen species (ROS) plays a critical role in glutamate-, β-amylloid-, and nerve growth factor- or serum-deprivation-induced apoptosis (Greenlund et al., 1995; Reynolds and Hastings, 1995; Butterfeld, 1997). Apoptosis associated with increased generation of ROS has been demonstrated by direct exposure of neuronal cells to oxidative stress, such as hydrogen peroxide, lipid hydroperoxide, and superoxide anion (Aoshima et al., 1997; Satoh et al., 1997). In the present study, xanthine (100 μM)/xanthine oxidase (150 μU/ml) was used as a ROS producer to induce apoptosis in PC12 cells. The ROS-induced changes in c-Myc, p53, and Bcl-2 family expression and caspase-1 and -3 activities were measured. At the same time, the effects of bilobalide, the main constituent of the nonflavone fraction of EGB; on the ROS-induced apoptosis; the expression of c-Myc, p53, and Bcl-2 family; and the caspase-1 and -3 activities were studied.

**Materials and Methods**

**Cell Culture.** PC12 cells were cultured at 37°C in a humidified CO₂ (5%) incubator in Dulbecco’s modified Eagle’s medium (Life Technologies, Gaithersburg, MD) supplemented with fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 U/ml). Twenty-four hours before addition of various agents, the cells were seeded on 6-well plates (Costar, Cambridge, MA) at a density of 10⁵ cells/cm² in serum-free Dulbecco’s modified Eagle’s medium containing transferrin (5 μg/ml), insulin (5 μg/ml), and progesterone (20 nM; Sigma Chemical Co., St. Louis, MO). Xanthine (100 μM)/xanthine oxidase (150 μU/ml) (Sigma Chemical Co.) was used to induce apoptosis. When the effect of bilobalide on the cells was studied, different concentrations of bilobalide and xanthine (100 μM/xanthine oxidase (150 μU/ml) were added simultaneously to the medium. Bilobalide was provided by Zhong-Liang Chen (Department of Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences). The purity of this compound was 98% (HPLC) and its electronic impact mass spectrum and 1H NMR spectrum were measured. DNA was purified with 2 volumes of ethanol in the presence of 0.3 M sodium acetate. After centrifugation at 12,000g for 15 min, the DNA pellets were washed with 70% ethanol, air-dried, and resuspended in 20 μl TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). DNA was separated on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide and photographed by UV transillumination.

**Flow Cytometric Analysis of DNA Content.** Cells were collected by centrifugation, washed with PBS, and fixed with 70% ethanol. The fixed cells were harvested by centrifugation at 200g for 10 min and resuspended in 100 μl of PBS containing 50 μg/ml RNase A, then incubated at 37°C for 1 h. After incubation, the cells were stained with 200 μg/ml propidium iodide (Sigma Chemical Co.) at 4°C for 30 min. The fluorescence of cell was measured with FACScan (Becton Dickinson FACStar Plus flow cytometer).

**Flow Cytometric Analysis of c-Myc, p53, Bel-2, Bel-xL, and Bax Protein.** The level of c-Myc, p53, Bel-2, Bel-xL, and Bax protein was measured by flow cytometry as described in Liu and Zhu (1999). Briefly, cells were collected by centrifugation and washed with PBS. After fixation with 2% paraformaldehyde for 20 min and permeabilization with 0.5% Triton X-100, cells were incubated with primary antibodies for c-Myc, p53, Bel-2, Bel-xL, and Bax, respectively, for 45 min, followed by incubation with corresponding fluorescein isothiocyanate-conjugated secondary antibodies (Boster Company) for 30 min at room temperature in the dark. After the cells were washed with PBS, the antigen density was measured by using a Becton Dickinson FACStar Plus flow cytometer and the percentage of positive cells was determined. The primary antibodies used were mouse monoclonal antibodies against c-Myc (Dako, Glostrup, Denmark); goat polyclonal antibodies against p53 (N-19); rabbit polyclonal antibodies against Bel-2, Bel-xL, and Bax (Santa Cruz Biotechnology, Santa Cruz, CA). All these primary antibodies were used at 1:50 dilution.

**RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** Total RNA was isolated from the cells with TRI Reagent LS (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. For cDNA synthesis, 20 μl of RT mixture contained 3 μl (1 μg) of total RNA template, 2 μl of 10 μM dNTPs, 1 μl (1.6 μg) oligo(dT)₁₈ primer (Sangon, Shanghai, China), 1 μl (20 U) of RNase inhibitor (Promega, Madison, WI), 1 μl (20 U) of M-MulV reverse transcriptase (MBI, Vilnius, Lithuania), and 4 μl of 5× reaction buffer (MBI). The mixture was incubated at 37°C for 90 min, followed by heating the reaction mixture at 95°C for 10 min.

PCR primers were 5'-ATCTTCTCCTTCCAGCGTGA-3' (forward) and 5'-TGCAGCTGACTGACCTC-3' (reverse) for bcl-2 (accession no. L14680); 5'-CTCGAGAGGATGTGCTCTGA-3' (forward) and 5'-GAGGAACTCCGAGTTGCAG-3' (reverse) for bax (accession no. U32098); 5'-AGCAGGCGGATGCTGGTTGAA3'-3' (forward) and 5'-CGTCCTTCGGCTCCATT-3' (reverse) for bcl-xL (accession no. X82537); and 5'-TTGTCGCTATCGTTGAGCT-3' (forward) and 5'-AGCTCTCTAGTGGCTGGTACGG-3' (reverse) for GAPDH (accession no. M17701). In PC12 cells, the expected sizes of the generated fragments are 386 base pairs (bp) for bcl-2, 206 bp for bax, 337 bp for bcl-xL, and 292 bp for GAPDH, respectively. One microliter of each RT solution was added to the PCR mixture containing 5 μl of 10× buffer, 2 μl of 2 mM dNTPs, 1 μl (10 pmol) each of the primers, and 0.75 μl (3.5 U) Taq DNA polymerase (MBI) in a total volume of 50 μl. PCR reactions were performed with a thermal cycler PTC-150 (MJ Research, Cambridge, MA). The cDNA was initially denatured for 2 min at 94°C followed by a 45-s denaturation at 94°C, a 1-min annealing at 55°C, a 2-min extension at 72°C, and finally, a 10-min extension at 72°C. In preliminary experiments, different numbers of cycles were performed to obtain data within the linear range of PCR amplification. PCR products were separated by 8% polyacrylamide gel electrophoresis and visualized by silver staining.
Caspase Cleavage Assay. Caspase activities were measured as described in Ochu et al. (1998) with minor modification. In brief, cells were washed with PBS and suspended in 500 μl of lysis buffer [0.5% Nonidet P-40; 10 mM HEPES, pH 7.4; 2 mM EDTA; 0.5 mM phenylmethylsulfonyl fluoride; and 5 μg/ml leupeptin (Sigma Chemical Co.), and allowed to lyse at 4°C for 30 min. Lysates were centrifuged at 7200g for 10 min, and the supernatant containing 50 μg of protein was incubated with 50 μM enzyme substrates (Ac-YVAD-AMC for caspase-1 and Ac-DEVD-AMC for caspase-3) (Calbiochem, La Jolla, CA) at 37°C for 30 min. Fluorescence was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm in a fluorescence spectrophotometer (Hitachi, 650-10s). The enzyme activity was expressed as fluorescent units per minute per milligram of protein. The protein concentration of the supernatant was determined by the method of Bradford (1976).

Statistical Analysis. Data are presented as mean ± S.E. Statistical analysis of the data for multiple comparisons was performed by ANOVA followed by Dunnett’s test. For single comparison, the significance of differences between means was determined by t test. A value of P < .05 was considered statistically significant.

Results

ROS-Induced Apoptosis in PC12 Cells. In agreement with a previous report (Satoh et al., 1997), exposure of PC12 cells to ROS for 12 h resulted in a characteristic cleavage of DNA at intranucleosomal sites, a biochemical hallmark of apoptosis, as shown in Fig. 1B. In parallel with the results of TUNEL, the DNA laddering pattern was observed after the cells were treated for 12 h with ROS (Fig. 2, lane 2). When the apoptosis was analyzed quantitatively by flow cytometry, a significant increase in the apoptosis rate (from 2.2 to 49.7%) was found after PC12 cells were treated with ROS for 12 h (Fig. 3B, left).

When the percentage of positive cells staining for c-Myc or p53 was determined with flow cytometry, 8.4 and 6% of the cells showed positive staining for c-Myc and p53, respectively, in the control groups (Fig. 4, 0h). After PC12 cells were treated with ROS for 3 h, the percentage of cells with positive staining for c-Myc and p53 increased to 27.8 and 50.1%, respectively (Fig. 4, 3h). Both c-Myc and p53 levels returned to basal level at 6 h after the treatment. When the percentage of positive cells staining for Bax was measured, 45.9% cells in the control groups showed positive staining, reflecting endogenous expression of Bax in PC12 cells (Fig. 4, 0h). After ROS treatment, Bax level increased in a time-dependent manner. The increase became obvious after 6 h of treatment and continued to increase. The level reached a maximum after 12 h of treatment. No Bcl-2 protein was detected in either control or ROS-treated cells (data not shown). However, a low level of Bcl-xL was detected in the control cells. When PC12 cells were treated with ROS, no change in the level of Bcl-xL was detected (Fig. 4).

When the level of Bax mRNA was measured with semi-quantitative RT-PCR, an obvious elevation of Bax mRNA level was detected after 3 h of treatment with ROS. The elevation reached a maximum after 6 h of treatment and lasted at least for another 9 h (Fig. 5). No Bcl-2 mRNA was detected in either control or ROS-treated cells (data not shown). Although Bcl-xL mRNA was detected in PC12 cells, no change was detected after the cells were treated with ROS (Fig. 5).

As shown in Fig. 6, caspase-3-like activity was up-regulated after 6 h of ROS treatment. The caspase-3-like activity reached a maximum after 12 h of treatment. No change in caspase-1-like activity was detected in PC12 cells during 0 to 15 h of treatment with ROS.

Effects of Bilobalide on ROS-Induced Apoptosis in PC12 Cells. When PC12 cells were treated with ROS and bilobalide (25–100 μM) simultaneously, the percentage of apoptotic cells decreased dose dependently, whereas bilobalide alone at 100 μM/xanthine oxidase (150 mU/ml; B), or xanthine (100 μM)/xanthine oxidase (150 mU/ml) + bilobalide (50 μM; C) for 12 h. Original magnification, 200×.

Fig. 1. TUNEL staining of apoptotic PC12 cells. PC12 cells were cultured on poly(L-lysine)-coated slides and treated with vehicle (A), xanthine (100 μM)/xanthine oxidase (150 mU/ml; B), or xanthine (100 μM)/xanthine oxidase (150 mU/ml) + bilobalide (50 μM; C) for 12 h. Original magnification, 200×.
Table 1). The percentage of cells with positive staining for c-Myc and p53 decreased from 27.8 and 50.1 to 16.7 and 23.2%, respectively, when cells were treated with 25 μM bilobalide.

Because both Bax expression and caspase-3-like protease activity reached a maximum after 12 h of treatment with ROS, the influence of bilobalide on Bax protein and caspase-3-like protease activity were observed at this time point. Bilobalide (50 μM) effectively reduced the elevation of Bax mRNA (Fig. 5) and protein (Table 1) levels induced by 12 h of treatment with ROS. The ROS-induced elevation of caspase-3-like protease activity also was reduced when the cells were treated with ROS and bilobalide simultaneously (Fig. 7). To measure the possible direct effect of bilobalide on caspase-3-like protease activity, bilobalide was added directly to the reaction buffer. However, no inhibitory effect was found when bilobalide (up to 100 μM) was added (data not shown).

Discussion

In this study, we examined the effect of ROS on PC12 cells. In agreement with a previous study (Satoh et al., 1997), exposure of PC12 cells to ROS resulted in an apoptotic cleavage of DNA in PC12 cells as shown in the TUNEL (Fig. 1) and DNA laddering pattern (Fig. 2). When the apoptosis was analyzed quantitatively by flow cytometry, a significant increase in the apoptosis rate was detected after PC12 cells were treated with ROS (Fig. 3). The results indicate that apoptosis is involved in PC12 cell death after the cells were exposed to ROS.

To detect the temporal changes of the apoptosis-related genes in PC12 cells after ROS treatment, a flow cytometric analysis was performed. In control groups, 8.4 and 6% of the cells showed positive staining for c-Myc and p53, respectively. After PC12 cells were treated with ROS, a significant increase in the percentage of cells with positive staining for c-Myc or p53 was detected. The up-regulation of c-Myc and p53 appeared as early as 3 h and then returned to normal at 6 h after ROS treatment. The transient increase of c-Myc and p53 may suggest that these two proteins are important apoptosis regulatory factors in the early stage of ROS-induced apoptosis in PC12 cells. Although early studies have shown that c-Myc is an oncoprotein with potent inductive effects on cell proliferation, recent studies have shown that c-Myc also possess proapoptotic activity. A possible explanation of this discrepancy is that the tendency of cells to undergo apoptosis is a normal consequence of cell proliferation machinery. Cell proliferation and apoptotic pathways may be coupled. c-Myc may not itself trigger apoptosis but may act as a sensitizer to whichever trigger is extant (Evan and Littlewood, 1998; Staunton and Gaffney, 1998).

In this study, we found that a significant increase in Bax expression was induced after PC12 cells were treated with ROS for 6 h. A similar result was obtained when Bax mRNA level was measured by RT-PCR. However, no Bcl-2 protein

Fig. 2. Electrophoresis pattern of DNA fragmentation. PC12 cells were treated with xanthine (100 μM)/xanthine oxidase (150 mU/ml) or xanthine (100 μM)/xanthine oxidase (150 mU/ml) + bilobalide (50 μM) for 12 h. Lane 1, DNA marker [pGEM-7Zf(+)/HaeIII]; lane 2, xanthine/xanthine oxidase; lane 3, xanthine/xanthine oxidase + bilobalide; and lane 4, control.

Fig. 3. Quantitative determination of apoptosis by flow cytometric DNA analysis. PC12 cells were treated with xanthine (100 μM)/xanthine oxidase (150 mU/ml) and different concentrations of bilobalide for 12 h. Left, representative histogram: shaded profiles, subdiploid DNA content; and open profiles, normal DNA content. Control (A), xanthine/xanthine oxidase (B), bilobalide (C; 100 μM), and xanthine/xanthine oxidase + bilobalide (D–F; 25, 50, and 100 μM, respectively). Right, data are mean ± S.E. of three independent experiments. *P < .05, **P < .01 versus group treated with xanthine/xanthine oxidase alone.
was detected in either control or ROS-treated cells. This result was consistent with previous studies (Mah et al., 1993; Zhong et al., 1993). Because Bcl-xL, another protein known to prevent apoptosis, has been detected in PC12 cells (Maroto and Perez-Polo, 1997), the role of Bax/Bcl-xL interaction in ROS-induced apoptosis was observed in the present study. A low level of Bcl-xL protein was detected in control cells. However, no change was found in the level of Bcl-xL protein after the cells were treated with ROS. In our previous studies, no change in Bax protein level was found during glutamate-induced apoptosis in cultured cortical neurons (Liu and Zhu, 1999). Instead, a significant down-regulation of Bcl-2 was found after the cortical neurons were treated with glutamate. These results together with those of our present study suggest that different intracellular mechanisms are likely to be involved in the apoptosis induced by different stimuli or environmental conditions in different cells.

Caspase activation, especially caspase-1 and caspase-3 activation, plays a critical role in the apoptosis of neurons (Tamatani et al., 1998; Tenneti et al., 1998). In this study, we found that caspase-3 activity was activated in PC12 cells by ROS treatment, whereas caspase-1 activity did not show any change during the experimental time. These results suggest that caspase-3 might be one of the main effector proteins in ROS-induced PC12 cell death. This result is consistent with that of previous studies showing that caspase-3 plays an
TABLE 1
Effect of bilobalide on c-Myc, p53, Bcl-xL, and Bax protein expression in PC12 cells treated with xanthine (100 μM)/xanthine oxidase (150 mU/ml)
The percentage of cells with positive staining was determined with flow cytometric assay. The levels of c-Myc and p53 were observed after 3 h of treatment with xanthine/xanthine oxidase. The levels of Bcl-xL and Bax were measured after 12 h of treatment with xanthine/xanthine oxidase. Data are mean ± S.E. of three independent experiments.

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<th>Control</th>
<th>Xanthine/Xanthine Oxidase + Bilobalide</th>
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<td>50</td>
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<tr>
<td>c-Myc (3 h)</td>
<td>8.4 ± 6.9</td>
<td>24.6 ± 6.2</td>
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<td>p53 (3 h)</td>
<td>6 ± 3.4</td>
<td>42.8 ± 7.3</td>
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<td>Bax (12 h)</td>
<td>45.9 ± 7.6</td>
<td>88.5 ± 6.1</td>
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<td>Bcl-xL (12 h)</td>
<td>3 ± 1.2</td>
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* P < .05, ** P < .01 versus group treated with xanthine/xanthine oxidase alone.

provided the first direct evidence showing that bilobalide could protect neurons from oxidative stress and that bilobalide might be working as a free-radical scavenger.

In this study, the effects of bilobalide on the apoptosis-regulatory and apoptosis-effector genes were further observed in PC12 cells. We found that bilobalide effectively reduced ROS-induced elevation of p53 and c-Myc in PC12 cells. We also demonstrated that bilobalide could antagonize ROS-induced up-regulation of Bax. Because p53 is a direct transcriptional activator of bax gene (Miyaushita and Reed, 1995), we were not surprised that both p53 and Bax were inhibited by bilobalide, although a direct inhibitory effect of bilobalide on Bax expression could not be excluded at present. The activation of caspase-3 has been suggested to be a downstream event of apoptosis. In this study, we found that ROS induced an elevation of caspase-3-like protease activity in PC12 cells and that this elevation was reduced when the cells were treated with bilobalide for 12 h (Fig. 7). However, the molecular target of bilobalide is not caspase-3 itself because the addition of bilobalide (up to 100 μM) directly to the reaction buffer did not show any effect on caspase-3 activity. These results suggest that bilobalide may block apoptosis in its early stage.

In summary, our results demonstrated that apoptosis is involved in ROS-induced PC12 cell death and that p53, c-Myc, and Bax expression and activation of caspase-3 play important roles in apoptosis. Among them, the up-regulation of c-Myc and p53 play important roles in the early stage of apoptosis. The transient up-regulation of p53 and c-Myc may cause the elevation of Bax expression and the activation of caspase-3. Our results further demonstrated that bilobalide could attenuate ROS-induced apoptosis, suggesting that bilobalide might be working as a free-radical scavenger. Bilobalide may block apoptosis in the early stage and then attenuate the elevation of c-Myc, p53, and Bax and activation of caspase-3 in cells.

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