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Research Article

The JNK/AP1/ATF2 pathway is involved in H_2O_2-induced acetylcholinesterase expression during apoptosis

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Abstract. We show that H_2O_2 increases acetylcholinesterase (AChE) expression *via* transcriptional activation through c-Jun N-terminal kinase (JNK), since the JNK inhibitor SP600125, but not the extracellular signal-regulated kinase (ERK) pathway inhibitor PD98059 or p38 kinase inhibitor SB203580, attenuated H_2O_2-induced AChE expression and its promoter activity. Overexpression of hemagglutinin (HA)-JNK increases H_2O_2-induced AChE expression and its promoter activity, whereas the dominant negative mutant form of JNK suppressed H_2O_2-induced AChE expression and promoter activity. Mutation analysis indicates that the major response elements for JNK in the AChE promoter are the AP1–like element (TGAGTCT) site, located within the -1565/-1569 region of the AChE promoter, and the ATF2 element (CCACGTCA), within the -2185/-2177 region. The AP1-like element binds to the transcription factors, c-jun and ATF2, while the ATF2 element binds mainly ATF2. Taken together, our results strongly suggest that H_2O_2 induces AChE expression *via* the JNK/AP1/ATF2 signaling pathway.

Keywords. JNK/AP1/ATF2, acetylcholinesterase expression, apoptosis, free radicals.

Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a carboxylesterase that plays an essential role in acetylcholine-mediated neurotransmission [1, 2]. AChE is expressed in the nervous system and muscle, where it hydrolyzes acetylcholine to terminate cholinergic neurotransmission [1, 3]. AChE has a short C-terminal peptide sequence that defines the post-translational processing and targeting of the enzyme. Differences in this peptide sequence define three isoforms of AChE: AChE-S (tailed isoform), AChE-E (erythtocytic isoform) and AChE-R (read-through isoform) [4].
Recently, several reports have shown that AChE is involved in many non-cholinergic functions, such as cell adhesion, proliferation, neurite outgrowth and regulation of apoptosis [5–9]. Previously, we have shown that upon induction of apoptosis, AChE is expressed in a number of non-muscle, non-nervous and non-hematopoietic cell lines [10–12]. Moreover, it has been demonstrated that AChE exerts a pivotal role in apoptosis by participating in the formation of the apoptosome [13]. Although it has been reported that AChE expression is regulated by calcium signaling during apoptosis induced by calcium ionophore

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A23187 and thapsigargin [11, 14, 15], the exact mechanisms that regulate AChE expression during apoptosis remain unclear.

Mitogen-activated protein kinases (MAPKs) constitute a widely distributed family of serine/threonine protein kinases that has been highly conserved during evolution. MAPKs are activated through dual phosphorylation of a specific threonine and tyrosine residue by the MAPK kinases (MKKs) [16]. There are three well-characterized MAPK subfamilies: the extracellular signal-regulated kinases (ERKs), the c-Jun-N-terminal kinases (JNKs) and p38-MAPK [16, 17]. MAPKs have been implicated in the regulation of cell apoptosis and cell growth arrest [18, 19], and the dynamic balance between ERK, JNK and p38-MAPK pathways are important in determining whether a cell survives or undergoes apoptosis [20, 21]. Phosphorylation and activation of MAPKs are essential for H_2O_2-induced apoptosis in various cell types [22–24]. Furthermore, inhibitors of MAPKs have been shown to significantly reduce H_2O_2-induced cell apoptosis [18, 25]. Interestingly, H_2O_2 has been shown to induce AChE activity in PC12 pheochromocytoma cells [26], and during anticancer drug-induced apoptosis, AChE induction was mediated by the JNK pathway [18]. Thus, we hypothesize that during apoptosis, AChE expression is related to oxidative stress and activation of the MAPK pathway.

In the promoter region of the AChE gene, there are many transcription factor binding sites such as the AP2 element, SP1 element and ATF/CREB element [1]. The downstream targets of the MAPKs also include a number of transcription factors including members of the MEF2 family, c-Jun, the ATF family and Elk [16, 17, 27]. We examined whether H_2O_2-induced AChE expression and activation was regulated by the MAPK pathway *via* transcriptional activation.

In summary, we found that the expression of AChE was markedly increased in H_2O_2-induced apoptosis in 293T cells, and that AChE expression during H_2O_2-induced apoptosis involved the JNK/AP1/ATF2 pathway. Thus, we propose that the oxidative-stress signal can be potentially transduced to the nucleosome *via* the activator protein-1 (AP-1) components: ATF2 and c-Jun.

Materials and methods

Cell culture and treatment. The 293T cells were maintained in DMEM (GIBCO-BRL, Gaithersburg, MD, USA) with 10% heat-inactivated fetal bovine serum (GIBCO-BRL). Apoptosis was induced by H_2O_2 (500 μM) (Sigma, St Louis, MO, USA) in 293T cells, and assessed by DNA fragmentation and Western blot analysis of cleaved caspase-3 (Santa Cruz Biotechnology, CA, USA). The inhibitors of ERK (PD98059, 30 μM), p38 (SB203580, 10 μM)) and JNK (SP600125, 20 μM) were purchased from Calbiochem. They were dissolved in DMSO.

Antibodies. Rabbit polyclonal antibodies specific for anti-cleaved caspase-3 antibody, rabbit polyclonal anti-JNK antibody, and mouse monoclonal anti-phosphorylated JNK antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). The AChE-specific antibody was from BD Biosciences (San Jose, CA, USA). The monoclonal anti-β-actin antibody was from Sigma and the horseradish peroxidase (HRP)-conjugated secondary antibody was from Santa Cruz Biotechnology. Goat polyclonal anti-p-c-jun antibody, goat polyclonal anti-p-ATF2 antibody and rabbit polyclonal anti-Smad4 antibody were purchased from Santa Cruz Biotechnology.

Determination of AChE activity. Relative AChE activity was determined spectrophotometrically in a 96-well plate using a modified Ellman's assay as described previously [28], and normalized against total protein. Cells were suspended in extraction buffer (50 mM potassium phosphate, pH 7.4, 1 M NaCl, 0.5% Tween-20). Lysates were clarified by centrifugation for 10 min at 4°C, and the protein concentration in the supernatants was assayed using a Bicinchoninic acid (BCA) method. Relative AChE activity was measured with an equal amount of total protein. Tetraisopropyl pyrophosphoreonicle (iso-OMPA, 0.75 mM) was added to exclude possible BuChE activity. The relative AChE activity values were expressed as a multiple relative to untreated control cells (defined as =1 U).

Semiquantitative RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and was subjected to reverse transcription using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Reverse transcription products were used for semiquantitative RT-PCR with oligonucleotide pairs specific for AChE and GAPDH. The primer pair (5′-CGG GTC TAC GCC TAC GTC TTT GAA CAC CGT GCT TC-3′; 5′-CAC AGG TCT GAG CAG CGA TCC TGC TTG CTG-3′) was designed to generate a 482-bp fragment of the tailed AChE-T mRNA, including exons E1–E2–E3–E4–E6, according to *Homo sapiens* AChE (YT blood group) (ACHE), transcript variant E4–E6, mRNA, NM_000665. Primers for the housekeeping gene, GAPDH (5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′; 5′-TCT AGA CGG CAG GTC AGG TCC

ACC-3′) were used as an internal control, with an expected product size of 588 bp.

Western blot analysis. Equal numbers of cells were lysed in 1 % NP-40, 50 mM HEPES (pH 7.4), 150 mM NaCl, 500 μM orthovanadate, 50 mM $ZnCl_2$, 2 mM EDTA, 2 mM phenylmethylsufonyl fluoride, 0.1 % SDS, and 0.1 % deoxycholate. Samples were incubated at 4°C for 10 min and then centrifuged at 10 000 *g* for 15 min at 4°C. SDS sample buffer was added and supernatants were mixed and boiled in SDS sample buffer. The lysates were separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was then incubated at room temperature in a blocking solution composed of 5 % skim milk powder dissolved in 10 mM Tris pH 8.0), 140 mM NaCl, and 0.1 % Tween-20 (TBST) for 1 h followed by incubation with the blocking solution containing anti-human AChE at 1 μg/ml for 4 h at room temperature. The membrane was washed three times in TBS (5 min each), and then incubated with an HRP-conjugated protein A antibody in the blocking solution. After washing three times in TBS, the immunoreactive protein was visualized using a chemiluminescence detection kit (ECL, Santa Cruz Biotechnology).

Transient transfections and luciferase assays. The 2.2-kb DNA fragment of the human AChE promoter [1] was subcloned into the *Bgl*II and *Hin*dIII sites of the pGL3 basic vector (Promega) with a downstream tagged firefly luciferase gene, *pAChE-Luc* [29]. An AP1-like element mutation within the −1535 to −1529 fragment of the human AChE promoter was created using a PCR-based mutagenesis procedure with the following forward (F) and reverse (R) end primers: F, 5′-GTG AAT TCC CCT CCC CGC CCG-3′; and R, 5′-GGG AAT TCA CCT GAG GCG GCC-3′. The PCR product, including the AP1-like element mutation, was replaced in the wild-type pAChE-Luc with two *Kpn*I sites to construct the AP1-like element mutant. An ATF2 binding site mutation within the -2184/-2177 fragment of the human AChE promoter was created with the following primers: F, 5′-CCA GGA GTG TCC CTG GTC ACC-3′; and R, 5′-GCA CGG GTG CAG AAA AGG TGA CCA GGG-3′. The ATF2 binding mutation was incorporated into the wild-type pAChE-Luc to construct the ATF2 binding mutant. All PCR-derived constructs were confirmed by sequencing.

293T cells were cotransfected using LipofectAMINE reagent (Invitrogen) with either the firefly luciferase reporter construct pAChE-Luc, AP1-like element mutant or ATF2 binding site mutant, together with the internal control, *Renilla* luciferase reporter plasmid pRL-SV40 (Promega). Apoptosis was induced by addition of H_2O_2, 24 h after transfection. Luciferase activity assays were performed with a Dual-Luciferase Reporter Assay System (Promega), and activity was measured with a luminometer BGP (MGM). Firefly luciferase activity was normalized to *Renilla* luciferase activity in each well (n=3/transfection experiment).

Electrophoretic mobility shift assays (EMSAs). Nuclear extracts of control and apoptotic 293T cells were prepared as described previously [30] with some modifications. EMSAs were performed with nuclear extracted protein (4 μg) in binding buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl, 0.5 mM DTT, 4 % glycerol, pH 7.5) and poly(dI:dC) (1 μg). Binding reactions were incubated (20 min, room temperature) with 20 000 cpm (0.1 – 0.5 ng) double-stranded oligonucleotides end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Invitrogen). An excess of unlabeled double-stranded oligonucleotides was used as competitors where indicated. Double-stranded oligonucleotides corresponding to the AP1-binding site were 5'-TCA GGT GAG TCT CCC TCC CCG CCC GG-3', named Wt AP1-like element. Mutant double-stranded oligonucleotides corresponding to the AP1-like element were 5'-TCA GGT GAA TTC CCC TCC CCG CCC GG-3', named Mut AP1-like element. The consensus sequences of the AP1 were as follows: AP1 (5'-CGC TTG ATG ACT CAG CCG GAA-3') (sc-2501; Santa Cruz Biotechnology). Mutant oligonucleotides corresponding to the consensus binding site for the AP1 transcription factor were (AP1mut) (5'-CGC TTG ATG ACT TGG CCG GAA-3') (sc-2514; Santa Cruz Biotechnology). Double-stranded oligonucleotides corresponding to the ATF2 binding site were 5'-CCA GGA GTG TCC CAC GTC ACC-3'. Mutant oligonucleotides of the binding site for the ATF2 transcription factor were (5′-CCA GGA GTG TCC CTG GTC ACC-3′). The consensus sequences of the ATF2 were as follows: ATF2 (5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3') (sc-2504; Santa Cruz Biotechnology). Mutant oligonucleotides corresponding to the consensus binding site for the ATF2 transcription factor were 5'-CGC TTG ATG ACT TGG CCG GAA-3'. (sc-2517; Santa Cruz Biotechnology). Antibody supershift assays were carried out in the same buffer, with further addition of 2 μl anti-p-c-jun (sc-16312X), anti-p-ATF2 (sc-7982X) and anti-Smad4 (sc-13044X) antibodies (Santa Cruz Biotechnology). The reaction was electrophoresed on a 4 %, 0.5× TBE native polyacrylamide gel then autoradiographed to detect specific DNA–protein complex formation.

Chromatin immunoprecipitation assay. Assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotech, Lake Placid, NY, USA) according to the manufacturer's protocol. Cells were fixed with 1% formaldehyde (15 min, 37°C). Cell pellets were resuspended in SDS lysis buffer and sonicated using an ultrasonic homogenizer VP-5S (TAITEC, Tokyo, Japan). After centrifugation, the supernatant was diluted in chromatin immunoprecipitation dilution buffer then incubated (overnight, 4°C) with anti-p-c-jun antibody and anti-p-ATF2 antibody (Santa Cruz Biotechnology). Immune complexes were recovered by the addition of 60 µl salmon sperm DNA/protein A-agarose slurry, followed by incubation (2 h, 4°C). Beads were washed with both low and high salt buffers, a LiCl buffer and a Tris/EDTA buffer, and immune complexes were eluted with an elution buffer (1% SDS, 100 mM $NaHCO_3$, and 1 mM dithiothreitol). To reverse DNA cross-linking, eluates were added to 5 M NaCl (8 µl) and incubated (4 h, 65°C), followed by treatment with proteinase K. DNA was recovered by phenol-chloroform extraction and ethanol precipitation and pellets were resuspended in 50 µl Tris/EDTA buffer. PCR was carried out for 35 cycles using 5 µl sample DNA solution, and PCR products were separated on 2% agarose gels in 1× Tris–acetate/EDTA.

Statistical analyses. Each experiment was repeated a minimum of three times. Results were expressed as means ± SEM and compared using Student's two-tailed *t*-test: * $p<0.05$ and ** $p<0.005$ were calculated using Student's two-tailed *t*-test.

Results

AChE expression in 293T cells was increased during H_2O_2-induced apoptosis. We confirmed that H_2O_2 induces apoptosis in 293T cells. Addition of H_2O_2 resulted in a dose-dependent appearance of ladder-like DNA fragments and caspase-3 cleavage in a time-dependent manner (data not shown). Western blot analysis revealed up-regulation of AChE expression by H_2O_2. Furthermore, a 2.5- to 3-fold increase in AChE activity was observed after exposure to H_2O_2 for 48 h. RT-PCR analysis revealed that AChE mRNA levels were significantly increased in H_2O_2-treated 293T cells.

JNK1/2 is involved in the induction of AChE by H_2O_2. To elucidate the signal transduction pathway by which H_2O_2 stimulates AChE expression, we used chemical inhibitors targeting ERK (PD98059), p38 kinase (SB203580) and JNK (SP600125). The 293T cells were pre-incubated with PD98059 (30 µM), SB203580 (10 µM) or SP600125 (20 µM) for 2 h, then were exposed to H_2O_2 (500 µm) for 24 h, finally the AChE activity was determined. As shown in Figure 1A, H_2O_2 induced a 60–70% increase in AChE activity above the low basal level. Inhibition of the JNK pathway using SP600125 almost completely suppressed H_2O_2-induced AChE activity, while PD98059 and SB203580, specific for ERK and p38 kinase, respectively, inhibited AChE activity by approximately 30–40% (Fig. 1A). Data obtained from the Western blot analysis (Fig. 1B) showed that SP600125 suppressed H_2O_2-induced AChE protein expression, whereas PD98059 and SB203580 were less effective. In addition, SP600125 abrogated the H_2O_2-induced increase in AChE mRNA (Fig. 1C).

JNK1/2 activity was assessed by examining the phosphorylation status of JNK1/2. We found that JNK1/2 activity was increased in 293T cells treated with H_2O_2 (Fig. 1D). We used dominant negative forms of JNK1 and JNK2 to suppress JNK activity and to examine its effect on the induction of AChE activity by H_2O_2. As shown in Figure 1E, transfection of dominant negative JNK1/2 reduced AChE activity. Conversely, overexpression of hemagglutinin (HA)-JNK1/2 stimulated H_2O_2-induced AChE activity (Fig. 1F). These results suggest that H_2O_2 stimulates AChE expression at least in part *via* the JNK signaling pathway.

H_2O_2 increases expression of AChE *via* transcriptional activation. We examined whether the induction of AChE following oxidative stress is the result of transcriptional activation. Assay of the promoter activity demonstrated that AChE expression was up-regulated *via* transcriptional activation following addition of H_2O_2 (Fig. 2A). The JNK pathway inhibitor, SP600125, suppressed the H_2O_2-induced increase in AChE promoter activity (Fig. 2B). Furthermore, transfection with dominant negative JNK1/2 reverted the H_2O_2-induced increase in AChE promoter activity (Fig. 2C).

AP1-like element (ALE) and ATF2 element play important roles in the H_2O_2-induced increase in AChE. We next investigated the critical elements that mediate the stimulatory effect of H_2O_2 on AChE promoter activity. Our data suggest that JNK1/2 is involved in the induction of AChE by H_2O_2. The downstream targets of JNK1/2 include a number of transcription factors including c-Jun, c-fos and ATF2, and these proteins bind to the AP1 element or ATF2 element to regulate promoter activity. Thus, we hypothesized that H_2O_2 may stimulate AChE promoter activity *via* the AP1 or ATF2 sites.

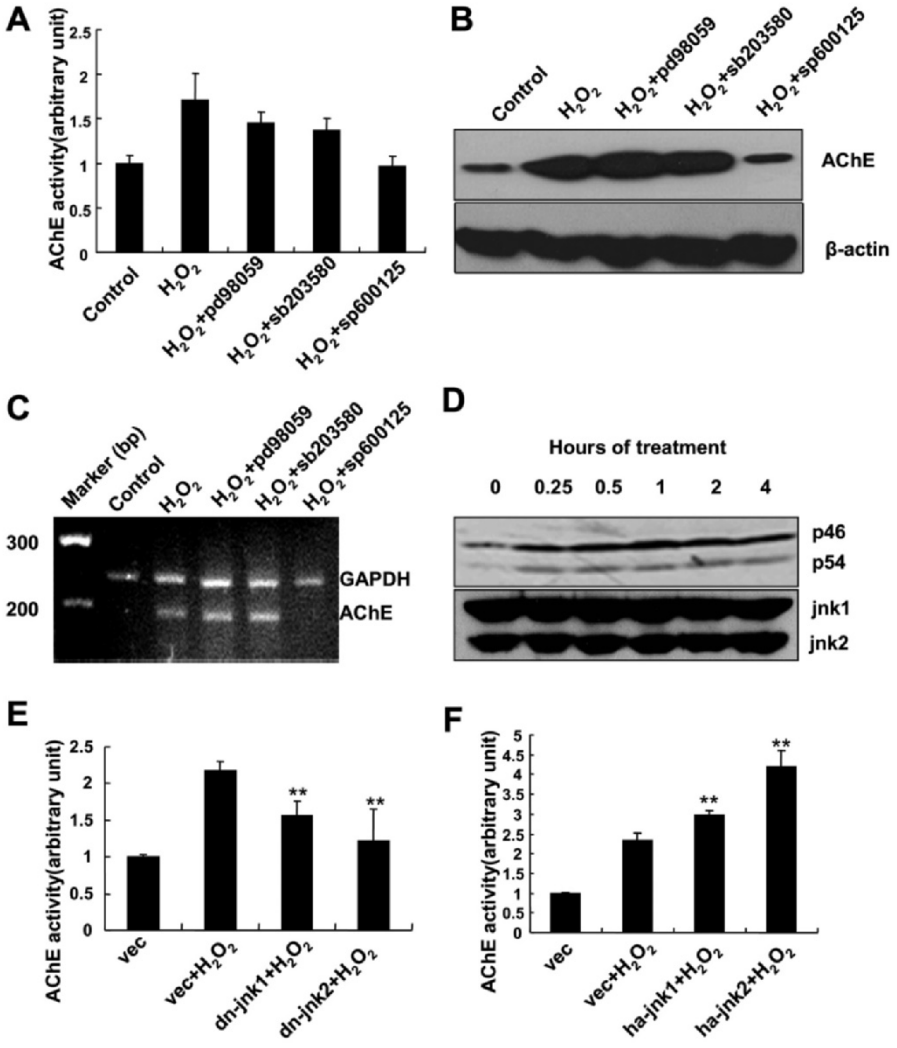

Figure 1. Effects of mitogen-activated protein kinases (MAPKs) on acetylcholinesterase (AChE) expression during H_2O_2-induced apoptosis in 293T cells. (*A*–*C*) Cells were pre-incubated with PD98059 (30 μM), SB203580 (10 μM) or SP600125 (20 μM) for 2 h prior to treatment with H_2O_2 (500 μM) for 24 h. (*A*) AChE activity was determined by the Ellman's method; $^{**}p<0.005$ relative to cells incubated with H_2O_2 (500 μM) only. (*B*) Western blot analysis was used to detect AChE protein levels. Actin served as the loading control. (*C*) RT-PCR was used to detect mRNA levels. GAPDH served as the internal control. (*D*) Cells were treated with H_2O_2 (500 μM) for the indicated time periods, and cell lysates were analyzed by Western blot with phospho-JNK and JNK antibodies. (*E*) Cells transfected with dominant negative JNK1 (dn-jnk1), dominant negative JNK2 (dn-jnk2) or vector alone were treated with H_2O_2 (500 μM) for 24 h. Changes in the H_2O_2-induced AChE activity after transfection were determined by Ellman's method; $^{**}p<0.005$ relative to H_2O_2-treated cells transfected with vector alone. (*F*) Cells transfected with hemagglutinin (HA)-JNK1 (ha-jnk1), HA-JNK2 (ha-jnk2) or vector alone, were treated as in (*E*).

Analysis of the region upstream of the AChE promoter did not reveal the presence of an AP1 consensus binding site. However, one AP1-like element (TGAGTCT) was observed in the -686/-692 region of the human AChE promoter. The AP1 consensus sequence has the inverted repeated sequence, TGACTCA. However, the AP1-like element (ALE) in the AChE promoter has an incomplete inverted repeated sequence, TGAGTCT. To determine whether the ALE is required for transcriptional activation of H_2O_2-induced AchE, we introduced a 4-bp mutation into the ALE. This mutation resulted in a CCGG to GGTT substitution at position -686 bp, thus eliminating the ALE and creating a novel *Eco*RI site (Fig. 3A). When the effect on resultant luciferase activity was examined in 293T cells, the ALE mutant showed little increase in luciferase activity in the presence of H_2O_2, in contrast to the 4-fold increase observed with the control construct (Fig. 3B).

Previous studies have reported that the AChE promoter includes one ATF-2/CREB(-2185/-2177) element. Mutations of the ATF2 site were also constructed (Fig. 3A) so as to further elucidate its role in H_2O_2-induced increases in AChE promoter activity. As shown in Figure 3C, in comparison to the wild-type construct, mutation of the ATF2 site largely prevented H_2O_2-induced AChE up-regulation, suggesting that this regulatory element might mediate the H_2O_2 response similar to the ALE.

Identification of the H_2O_2-responsive element within the promoter region of the ACHE gene. To investigate the H_2O_2-responsive element within the promoter region of the *ACHE* gene, ALE site probes were used in EMSA of nuclear extracts from 293T cells. While the ALE probe was found to form a complex with nuclear extracts from control cells (Fig. 4A, lane 2), nuclear extracts from apoptotic cells showed greater

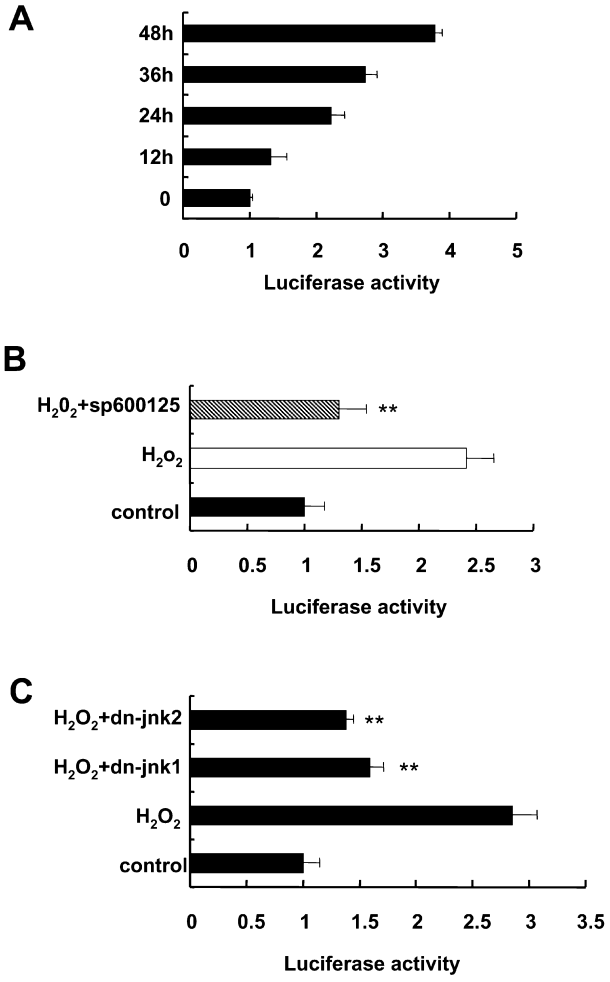

Figure 2. Effects of H_2O_2 on the AChE promoter activity during apoptosis in 293T cells. Cells were cotransfected with pAChE-Luc and pRL-SV40. (*A*) The transfectants were treated with H_2O_2 (500 μM) for different periods of time and AChE promoter activity was analyzed by luciferase activity. (*B*) Effects of the JNK inhibitor SP600125 on AChE promoter activity during H_2O_2-induced apoptosis. The transfectants were exposed to H_2O_2 (500 μM) for 24 h in the absence or presence of SP600125 (20 μM) and AChE promoter activity was analyzed by luciferase activity; $^{**}p<0.005$ relative to H_2O_2-treated cells in the absence of SP600125. (*C*) Dominant negative JNK1/2 suppresses H_2O_2-induced AChE promoter activity. The pAChE-Luc was co-transfected with control, dominant negative JNK1 (dn-jnk1) or dominant negative JNK2 (dn-jnk2) expression vectors into 293T cells then treated with H_2O_2 (500 μM) for 24 h. AChE promoter activity was assayed and normalized by luciferase activity. $^{**}p<0.005$ relative to H_2O_2-treated cells transfected with vector alone.

binding (Fig. 4A, lane 6). An excess of unlabeled probe incorporating the mutation in the ALE did not inhibit the formation of complexes between the labeled ALE probe and normal or apoptotic nuclear extracts (Fig. 4A, lanes 4 and 8). However, an excess of unlabeled probe, specific for the ALE competed for complexes formed by the labeled ALE probe with both the normal and apoptotic nuclear extracts (Fig. 4A, lanes 3 and 7). We also performed EMSA using oligonucleotides containing the consensus AP1 element or mutated AP1 element as competitors. The DNA-protein complex that was clearly visualized using probes for the ALE was eliminated by the addition of unlabeled consensus AP1 oligonucleotide, but unaffected when the mutated AP1 element was used as the competitor (Fig. 4C, lanes 2–4). We next examined the ATF-2 binding site within the AChE promoter using the same strategy as for the ALE site and nearly identical results were obtained (Fig. 4B, D).

Identification of the transcription factors binding to the ALE and ATF-2 element. To identify proteins able to bind to the ALE, antibodies against c-jun and p-ATF2 were used in EMSA. As shown in Figure 5A, both antibodies inhibited the DNA-protein complex to some extent (Fig. 5A, lanes 4, 5), with slightly better inhibition by anti-c-jun. The ATF2 element binding proteins were similarly analyzed with nearly identical results (Fig. 5B), except inhibition was more evident with anti-p-ATF2 than with anti-p-c-jun. An antibody against Smad4 failed to inhibit binding to either ALE or ATF2 element.

We subsequently performed chromatin immunoprecipitation (ChIP) analysis to examine whether c-jun was able to bind to the AChE promoter. Protein–DNA complexes were immunoprecipitated with antibodies, and the recovered DNA fragments, including the ALE, were monitored by PCR using primers for the -1565/-1569 region of the AChE promoter. The primers flanking the AP1-like element of AChE promoter showed slight PCR product in control 293T cells (Fig. 5C, lane 3). In contrast, H_2O_2-treated cells had a large increase in PCR product (Fig. 5C, lane 5). Genomic DNA was used as a positive control for PCR (Fig. 5D, lane 1), while the absence of anti-p-c-jun antibody served as the negative control for ChIP (Fig. 5C, lanes 2, 4). These findings are in agreement with the EMSA results (Fig. 5A) and indicate that c-jun is able to bind directly to the ALE in the AChE promoter. Similar ChIP analysis of ATF2 binding to the AChE promoter was performed in the presence or absence of anti-ATF2 antibody (Fig. 5D) and revealed that ATF2 is able to bind directly to the ATF2 binding site in the AChE promoter.

Discussion

We examined whether AChE expression was activated through the JNK pathway during H_2O_2-induced apoptosis. Using a luciferase reporter we were able to identify regulatory elements that are acted upon in response to H_2O_2 treatment. These results show that:

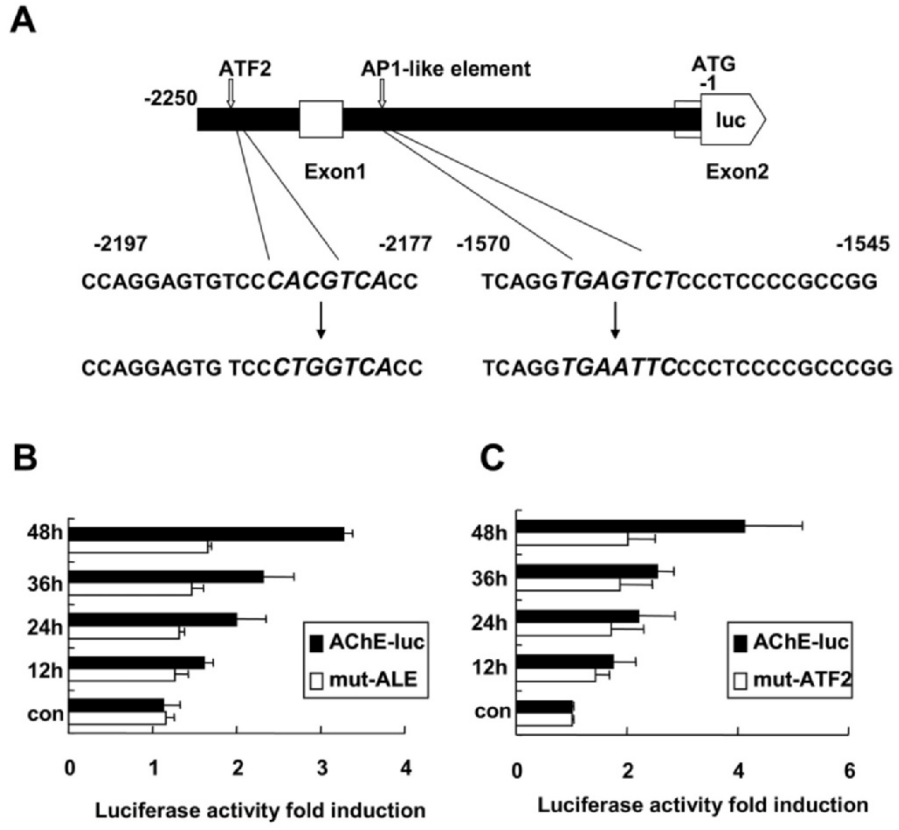

Figure 3. (*A*) The AP1-like element (ALE) within the -686/-692 fragment of the AChE promoter and the ATF2 element within the -2184/-2177 fragment of the AChE promoter are H_2O_2-responsive elements. The ALE mutant and the ATF2 element mutant from the AChE promoter were constructed as described in the materials and methods. (*B*) Wild-type and mutated human AChE promoters were tagged with the reporter luciferase, pAChE-Luc and ALE mutant, respectively. Cells were cotransfected with either pAChE-Luc or the ALE mutant, and pRL-SV40. The transfectants were treated with or without H_2O_2 (500 μM) for 24 h. AChE promoter activity was analyzed by luciferase activity. Values are given as the fold induction relative to untreated controls. (*C*) The ATF2 element was treated as in (*B*).

(i) AChE expression is increased during H_2O_2-induced apoptosis in 293T cells; (ii) JNK1/2 is involved in the induction of AChE by H_2O_2; and (iii) both the ALE and the ATF-2 element are involved in the H_2O_2-induced increase in AChE activity.

Previous studies have reported that AChE is expressed in various cell types treated with different inducers of apoptosis [10]. To date, however, the mechanism that regulates AChE expression during apoptosis has not been elucidated. Here, we examined the mechanisms involved in the H_2O_2-induced regulation of AChE during apoptosis. It has previously been suggested that H_2O_2 may activate ERK1/2, JNK1/2 and p38 in many cell lines [23, 31]. Here, we report that only the inhibitor of JNK1/2 (SP600125) down-regulated the activation of AChE induced by H_2O_2. Our results are consistent with a recent study by Deng et al. [18], which demonstrated that AChE expression is mediated by the JNK pathway during anti-cancer drug-induced apoptosis.

Our results show that AChE expression was induced *via* transcriptional activation following addition of H_2O_2. Thus, we studied further the effects of the *cis*-acting elements that mediate the stimulatory effect of H_2O_2 on AChE promoter activity. The AChE promoter has been shown to contain consensus recognition sites for transcription factors in various kinds of cells [1]. It has been reported that AChE promoter polymorphisms located 17 kb upstream of the AChE transcription start site identify a new HNF3-binding enhancer domain important for AChE expression [32]. Reports have demonstrated that human and mouse AChE genes contain at least four alternative first exons each, of which at least one encodes an extended N terminus [33]. Thus, regulation of AChE expression is very complicated and the AChE promoter activity is affected by many factors.

The downstream targets of JNK also include a number of transcription factors. ATF2 and c-jun have been previously shown to mediate oxidative stress-induced gene expression [34]. Thus, in an effort to assess potential substrates of JNK1/2 in H_2O_2-treated 293T cells, we first examined the ATF-2 and AP1 elements in the AChE promoter. Although, we did not identify a consensus AP1 element in the AChE promoter, we did find an ALE that contains a region that is highly homologous with the AP1 consensus sequence. Previous studies have shown that the ALE (TGACTCT) mediates transcription of the rat *p53* gene during liver regeneration. However, in this case, the protein binding to the ALE was not an AP1 family protein [35]. In the rat *vasopressin* gene, fos/jun proteins act as potent inducers of *vasopressin* gene transcription, partly *via* the ALE [36]. Our results suggest that c-jun

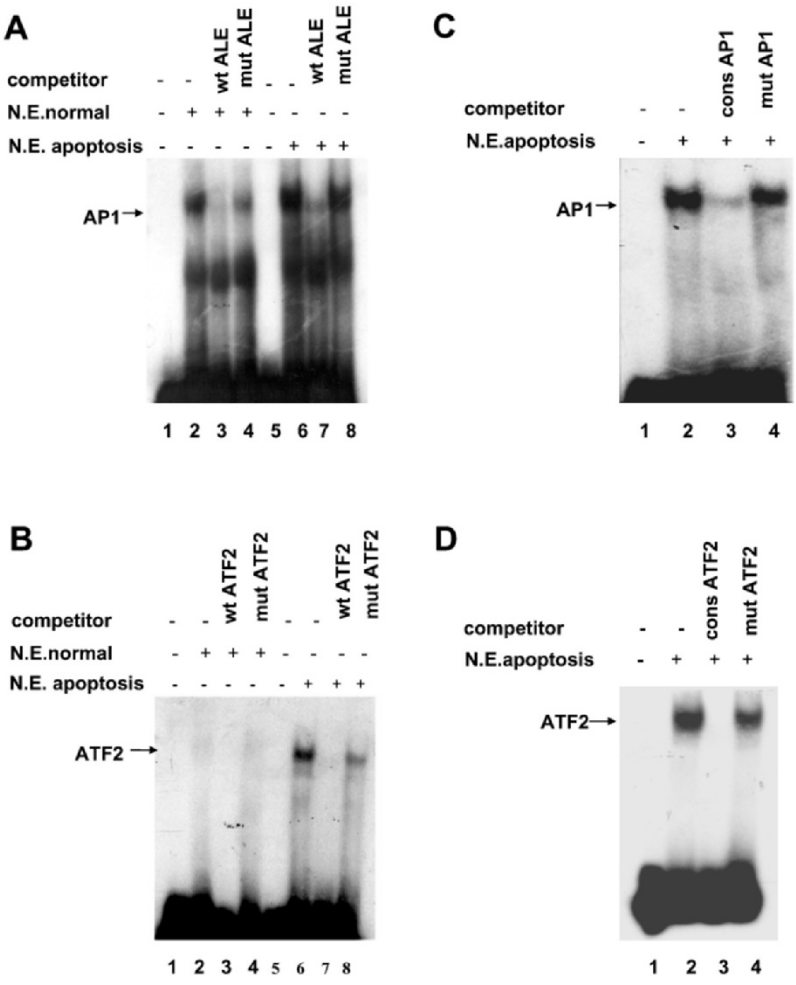

Figure 4. Identification of the H_2O_2-responsive element in the AChE promoter region. Cell apoptosis was induced by H_2O_2 (500 μM) for 24 h. (*A*) Interference assays using various consensus oligonucleotides as competitors. The ^{32}P-labeled ALE was incubated with nuclear extracts from normal (lanes 1–4) or apoptotic 293T cells (lanes 5–8) in the presence of the following competitors: the wild-type ALE (wt ALE; lanes 3 and7) and the mutated ALE (mut ALE; lanes 4 and 8). (*B*) The ^{32}P-labeled ATF-2 site was treated as in (*A*). (*C*) The ^{32}P-labeled ALE was incubated with nuclear extracts from apoptotic 293T cells (lane 2) in the presence of the following competitors: consensus AP1 sequence (consAP1; lane 3) and the mutated AP1 element (mutAP1; lane 4). (*D*) The ^{32}P-labeled ATF-2 site was treated as in (*C*). N.E.: nuclear extract.

and ATF2 proteins bind to the ALE in the *ACHE* gene promoter and regulate *ACHE* gene expression.

Previous studies have reported that the AChE promoter includes one ATF-2/CREB (-2185/-2177) element [1]. ATF2 has also been shown to be a target of the JNK signal transduction pathway that mediates transcriptional responses [37]. Also, cAMP-mediated AChE expression in chick muscle can be up-regulated by the ATF2 element [38]. Although the ATF2 element in general is a positive regulator, during myogenic differentiation of C2C12 cells, the ATF2 element in the human AChE promoter shows the opposite effect [39]. The muscle-specific suppressive response might be due to steric interference in the muscle-specific transcription machinery, such as the E-box motifs localized adjacent to the ATF2 element of the human AChE promoter [39]. Here, we show that ATF2 binds to the ATF2 element in the AChE promoter and up-regulates AChE expression in H_2O_2-treated 293T cells.

In summary, we show that in response to oxidative stress, AChE expression and activity is increased in 293T cells. This response is mediated by the JNK signaling pathway and involves the binding of transcription factors to the ALE and ATF2 element within the AChE promoter. Interestingly, oxidative stress has been widely implicated in the neuronal cell death associated with Alzheimer's disease (AD) [40–44]. Oxidative stress mediates the neurotoxic mechanisms of β-amyloid (Aβ), which are associated with neuronal loss in AD [45]. The appearance of phospho-JNK granules in the hippocampus is an early event in AD [46]. Previously, it has been shown that intracellular accumulation of Aβ triggers JNK activation in neurons of PS1-linked AD patients and transgenic mice carrying the mutant PS1 gene [47]. Studies in primary cultures revealed that Aβ-induced neuronal death is accompanied with JNK activation, suggesting that JNK activation may be an upstream event contributing to neuronal death in AD pathology [47]. Several *in vitro* and *in vivo* studies have reported that alterations of JNK pathways are potentially associated with neuronal death in AD, and intervention on the JNK pathway is considered to be a therapeutic

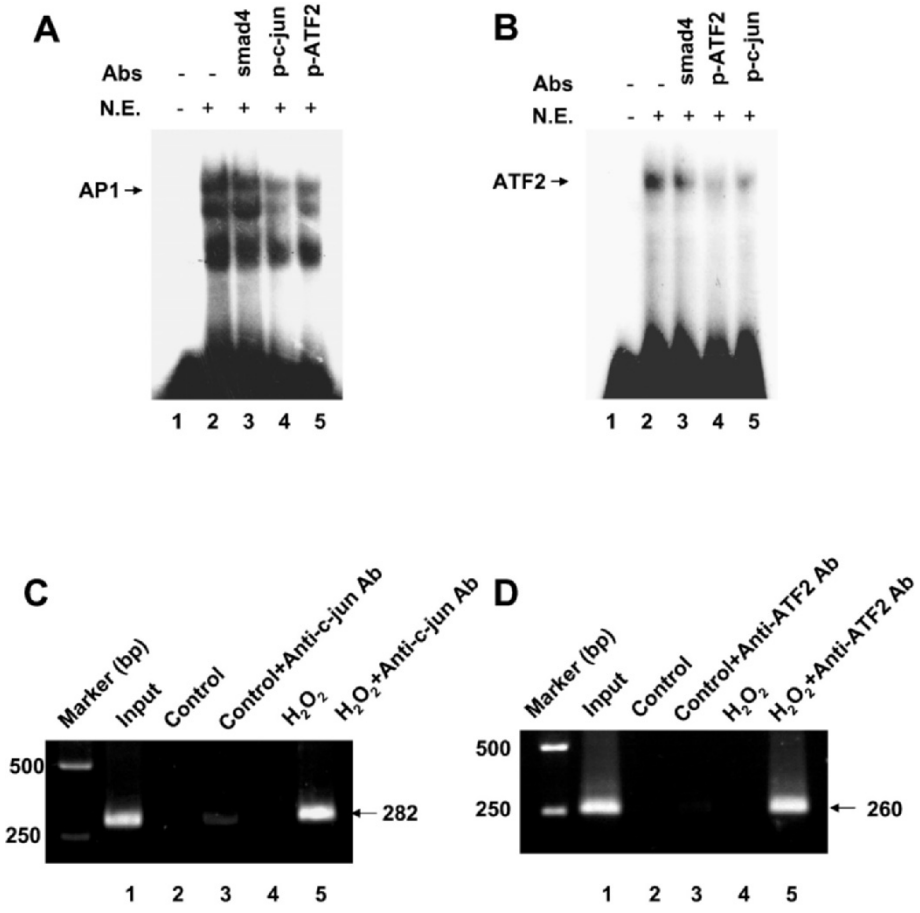

Figure 5. (*A*, *B*) Identification of the transcription factors binding to the ALE and ATF2 element. Interference assays using specific antibodies against transcription factors. Cell apoptosis was induced by H_2O_2 (500 μM) for 24 h. (*A*) The ^{32}P-labeled ALE was incubated with nuclear extracts from apoptotic 293T cells in the presence of various antibodies: anti-Smad4 (lane 3), anti-p-c-jun (lane 4) or anti-ATF2 (lane 5). (*B*) The ^{32}P-labeled ATF2 element was treated as in (*A*). (*C*) Chromatin immunoprecipitation analysis was performed to monitor the binding of c-jun to the AP1-like element within the -1565/-1569 fragment of the AChE promoter. Protein-DNA complexes were incubated with antibodies against p-c-jun and isolated by immunoprecipitation (lanes 3, 5). Positive controls were prepared before immunoprecipitation (lane 1) and negative controls were isolated by immunoprecipitation without antibody (lanes 2, 4). All immunoprecipitated DNA fragments were analyzed by PCR with the indicated primers. (*D*) Chromatin immunoprecipitation analysis was performed to monitor the binding of ATF2 to the ATF2 binding site within the -2185/-2177 fragment of the AChE promoter. Chromatin immunoprecipitation analysis of ATF-2 binding to the AChE promoter was performed using a method similar to that used in (*C*).

approach for AD [48]. Furthermore, AChE is a senile plaque component and the enzymatic activity of AChE is up-regulated in senile plaques and tangles during the process of amyloid deposition in AD [49, 50]. It has been shown that AChE increases Aβ assembly with the formation of highly toxic complexes (Aβ-AChE) [51]. The neurotoxic effect induced by Aβ-AChE complexes was higher than that induced by the Aβ peptide alone [51]. Since, we have shown that oxidative stress increases AChE expression *via* the JNK/AP1/ATF2 pathway, our findings may have some relevance to understanding the processes that contribute to the pathogenesis of AD.

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