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Histone Acetylase Inhibitor Trichostatin A Induces Acetylcholinesterase Expression and Protects Against Organophosphate Exposure

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Abstract The biological effects of organophosphorous (OP) chemical warfare nerve agents (CWNAs) are exerted by inhibition of acetylcholinesterase (AChE), which prevents the hydrolysis of the neurotransmitter acetylcholine, leading to hypercholinergy, seizures/status epilepticus, respiratory/cardiovascular failure, and potentially death. Current investigations show that bioscavenger therapy using purified fetal bovine AChE in rodents and non-human primates and the more recently tested human butyrylcholinesterase, is a promising treatment for protection against multiple LD_{50} CWNA exposures. Potential impediments, due to the complex structure of the enzyme, purification effort, resources, and cost have necessitated alternative approaches. Therefore, we investigated the effects of transcriptional inducers to enhance the expression of AChE to achieve sufficient protection against OP poisoning. Trichostatin A (TSA), an inhibitor of histone deacetylase that de-condenses the chromatin, thereby increasing the binding of transcription factors and mRNA synthesis, was evaluated for induction of AChE expression in various neuronal cell lines. Dose-response curves showed that a concentration of 333 nM TSA was optimal in inducing AChE expression. In Neuro-2A cells, TSA at 333 nM increased the extracellular AChE activity approximately 3–4 fold and intracellular enzyme activity 10-fold. Correlating with the AChE induction, TSA pre-treatment significantly protected the cells against exposure to the organophosphate diisopropylfluorophosphate, a surrogate for the chemical warfare agents soman and sarin. These studies indicate that transcriptional inducers such as TSA up-regulate AChE, which then can bioscavenge any organophosphates present, thereby protecting the cells from OP-induced cytotoxicity. In conclusion, transcriptional inducers are prospective new methods to protect against CWNA exposure. J. Cell. Biochem. 96: 839–849, 2005.

Key words: acetylcholinesterase; organophosphates; transcriptional inducers; neuronal cell lines; gene expression; neuroprotection

The biological effects of organophosphorous (OP) chemical warfare nerve agents are exerted by reversible or irreversible inhibition of acetylcholinesterase (AChE), which results in decreased catalyzed hydrolysis and excessive accumulation of the extracellular neurotransmitter acetylcholine (ACh). The ensuing hyperactivation of ACh receptors, including nicotinic and muscarinic receptors, results in various toxic effects involving hyper-secretions, convulsions, respiratory distress, coma, and ultimately death. Current emergency treatment of acute OP poisoning consists of combined administration of an AChE re-activator (an oxime), a muscarinic ACh receptor antagonist (e.g., atropine), and an anticonvulsant (e.g., diazepam) [Holstege et al., 1997; Broomfield and Kirby, 2001]. Recent investigations show that prophylactic enzyme therapy using purified AChE or human plasma butyrylcholinesterase (BChE) appears to be a promising pretreatment for OP exposure [Doctor et al., 1991; LeJeune et al., 1998]. Concerns associated with the complex structure of the cholinesterases,

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posttranslational modifications, genetic variations, intense labor, large amounts of human serum needed for purification, low yield of purified enzyme, potential viral contamination and the high-dose regimens required for therapy because of the stoichiometric interaction of the enzyme with the OP have necessitated alternative approaches to nerve agent bioscavenger pre-treatment. One novel approach is to induce the expression of endogenous cholinesterases using transcriptional inducers. In addition, transcriptional upregulation of cholinesterases is useful to produce large quantities of recombinant enzymes, which would preclude the requirement for human serum and thereby facilitate purification.

Accumulating evidence suggests that the cellular expression of AChE is regulated both transcriptionally and posttranscriptionally; the promoter of human AChE is activated by a cyclic AMP (cAMP)-dependent pathway and augmented by cAMP [Wan et al., 2000b; Siow et al., 2002]. Forskolin, a strong inducer of intracellular cAMP, also has been shown to induce AChE expression [Wan et al., 2000a]. Several binding sites of vitamin D3 and 17β-estradiol have been reported in human AChE promoters [Grisaru et al., 1999], and both of these factors enhance transcription of the AChE gene. Constitutively activated $G\alpha_s$ and activation transcription factor-1 showed 10- and 4-fold increase in AChE activity respectively [Wan et al., 2000a]. The RNA-binding protein HuD upregulates AChE posttranslationally by binding to the 3′ untranslated region [Deschenes-Furry et al., 2003]. Agonists of P2Y(1) receptors and peroxisome proliferator-activated receptor gamma strongly induce the expression of AChE in neuroblastoma cells [Choi et al., 1998, 2001; Han et al., 2001]. Neuronal–glial interactions mediated by interleukin-1 also significantly increase the expression of AChE [Li et al., 2000]. Taken together, these observations suggest that the constitutive expression of AChE can be significantly increased by treatment with transcriptional inducers.

We investigated whether the transcriptional enhancer, Trichostatin A (TSA), could upregulate the endogenous cholinesterases expressed in neuronal cell lines and whether the expressed cholinesterase could bioscavenge OPs. TSA inhibits histone deacetylase, leading to increased histone acetylation and decondensing of the chromatin [Arts et al., 1995; Finnin et al., 1999]. Chromatin decondensation enhances the binding of transcription factors and ultimately results in induction of gene expression. We demonstrate that TSA enhances the expression of both intracellular and extracellular AChE in neuroblastoma cell lines. Furthermore, the increased level of cholinesterases in TSA treated cells bioscavenge the OPs, and significantly protect the cells from the toxicity of organophosphate diisopropylfluorophosphate (DFP). These results indicate that transcriptional inducers of cholinesterases are potential new ways for prophylactic therapy against chemical warfare nerve agent exposure.

MATERIALS AND METHODS

Neuronal Cell Lines and Cell Culture

The mouse neuroblastoma cell line Neuro-2A (CCL-131) was obtained from American Type Culture Collection (Manassas, VA). Neuro-2A cell lines were cultured in minimum essential medium (Eagle) with 2 mM L-glutamine, penicillin–streptomycin, and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (FBS). The cells were cultured at 37°C in an incubator with humidified air and 5% CO_2.

Depletion of Cholinesterases From FBS

To minimize the interference during transcriptional induction and the enzyme assay, endogenous serum cholinesterase in the FBS were depleted using procainamide affinity chromatography [Ralston et al., 1983, 1985], which binds FBS AChE and BChE (butyrylcholinesterase). In brief, 10 ml procainamide gel was packed in a glass column and washed with 0.1 M phosphate buffer, pH 8.0. One hundred milliliters of FBS was loaded on to the column. The first 20 ml of the flow-through was discarded due to dilution with the phosphate buffer. The remaining 80 ml of the flow-through was collected and filter sterilized by passing it through 0.22–0.45 micron filters. Depletion of the serum AChE was confirmed by comparing the enzyme assays of the original and depleted FBS.

Analysis of the Effect of Transcriptional Inducers on AChE Expression

Cells were plated (1×10^5 cells/well/ml) in 48-well tissue-culture plates (Costar, Corning,

NY) using AChE depleted FBS medium. TSA (Sigma Chemical Co., St. Louis, MO) was diluted in AChE depleted medium and various doses of TSA were added to the cells and incubated for different time periods as indicated in each experiment. The cells were also incubated with repeated addition of TSA every other day for a total of 7 days. In some cases, TSA was added a single time at the start of incubation and in other cases TSA was added every other day for the entire incubation period.

AChE Microassay

AChE levels were determined by the Ellman's method that was adapted for 96-well plates [Ved et al., 1991; Dave et al., 1997]. For the AChE assay, an increase in absorbance was monitored at 412 nm for 10 min in a reaction mixture containing 20 μl extracellular or intracellular enzyme as described below, 10 μl of 30 mM acetylthiocholine iodide (ATC), 10 μL of 10 mM dithionitrobenzene, and 50 mM sodium phosphate buffer (pH 8) in a final volume of 300 μL.

Culture supernatant (20 μL) at the end of the incubation was used as the source of extracellular enzyme. For the estimation of intracellular AChE, at the end of the incubation, the medium was removed and the cells were washed with phosphate buffered saline (PBS) and lysed using ice-cold phosphate buffer containing 1% Nonidet P-40 or with a lysis buffer T-PER (Pierce Chemical Co., Rockford, IL). The homogenate was centrifuged for 10 min at 3,000*g*, 4°C, and the resulting supernatant was used for the intracellular enzyme assay.

Protein Determination

Total protein content of the cell lysate was determined by a BCA protein assay (Pierce Chemical Co.) using bovine serum albumin as a standard. Ten microliters of the lysate was added to 300 μl of reaction mixture and the optical density was measured at 562 nm in a microplate reader.

Karnovsky Staining of Cells for the Induction of AchE

The method of cytochemical staining was originally described by Karnovsky and Roots [1964] for the demonstration of AChE expression and was later modified [Hanker, 1975; Kobayashi et al., 1994]. In brief, cells in the 48-well plates were incubated for 15 min in a 300 μl reaction mixture prepared by the addition of 1.5 ml sodium phosphate buffer (pH 6), 10 mg acetylthiocholine iodide, 0.1 ml of 0.1 M sodium citrate solution, 0.2 ml of 30 mM copper sulphate solution, and 0.2 ml of 5 mM potassium ferricyanide solution at room temperature for 4–8 h. The cells were washed and observed with an Olympus IX51 microscope.

DFP Treatment and MTT Cytotoxicity Assay

Diisopropylfluorophosphate (DFP) (Sigma) was diluted in AChE-depleted medium, added to the cells, and incubated for 2–3 days. Neuronal cell survival was quantified by incubating the cells for 2–4 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which yields a blue formazan product in living cells but not in dead cells or their lytic debris [Mosmann, 1983]. The resulting colored product was solubilized in 70% isopropanol containing 2% HCl and the absorbance was measured at 620 nm using a microplate reader.

Cell Morphology Analysis by Microscopy

Changes in the cell morphology following transcriptional inducers or DFP was analyzed by microscopy using a Nikon microscope (20×).

Reverse Transcriptase Polymerase Chain Reaction

Two new AChE primers from the reported AChE mRNA sequence (Accession BC046327) (forward 1,325–1,358 bp, 5′GCT GTG GTC CTG CAT TAC ACA GAC 3′ and (reverse 1,899–1,874 bp, 5′TCC AGT GCA CCA TGT AGG AGC TCC AG 3′) were synthesized. Beta actin was used as a control and the primers were, forward 5′-CAT GGG TCA GAA GGA TTC CT-3′ and reverse 5′AGC TGG TAG CTC TTC TCC A3′. For AChE RT-PCR, RNA was isolated from Neuro 2A cells treated with or without different doses of TSA using the RNeasy mini kit (Qiagen, Santa Clarita, CA) according to the manufacturer's directions. Single stranded cDNA was synthesized from 0.2 to 1 μg total RNA using AMV reverse transcriptase-based reverse transcription system from Promega (Madison, WI) and oligo-dT primer as instructed by the manufacturer. Reverse transcription product was used for PCR amplification of expressed AChE or β actin control. The amplification was carried out using Taq polymerase and a Perkin-Elmer thermal cycler. The PCR amplification conditions are as follows: initial denaturation at 94°C

for 4 min; followed by 35 cycles at 94°C, 45 s; 55°C, 1 min: 72°C for 2 min: and a final extension at 72°C for 7 min. The PCR products (15 μl) were electrophoresed on 1.0% SeaKem agarose gel (FMC BioProducts, Rockland, ME) and visualized with ethidium bromide or the more sensitive Sybergreen staining.

RESULTS

Depletion of Serum AChE by Procainamide Gel Affinity Chromatography

To reduce the background interference of soluble AChE found in the FBS supplement and to determine proper expression of AChE following induction with transcriptional enhancers, the serum cholinesterases was depleted using procainamide gel affinity chromatography. Column chromatography, rather than batch incubation with the gel used for purifying AChE, was carried out to minimize the time of interaction between FBS and procainamide to prevent non-specific binding and elimination of any other serum factors (Fig. 1A). AChE present in the serum increases the background and could skew the results or exhibit feedback inhibition of enzyme expression. An enzyme microassay showed that the AChE was completely depleted from the FBS by this protocol (Fig. 1B). To further show that procainamide does not leech out from the column and inhibit the expressed AChE, we have incubated equal volumes of untreated FBS and procainamide treated FBS and assayed the AChE activity. As shown in Figure 1C, the AChE value of the mixture was half the value of the untreated serum demonstrating that procainamide did not leech out from the column and inhibit the enzyme during the assay. Protein estimation results showed no significant decrease in the level of protein after depletion of cholinesterases indicating that the removal of non-specific proteins was minimal following procainamide gel affinity chromatography.

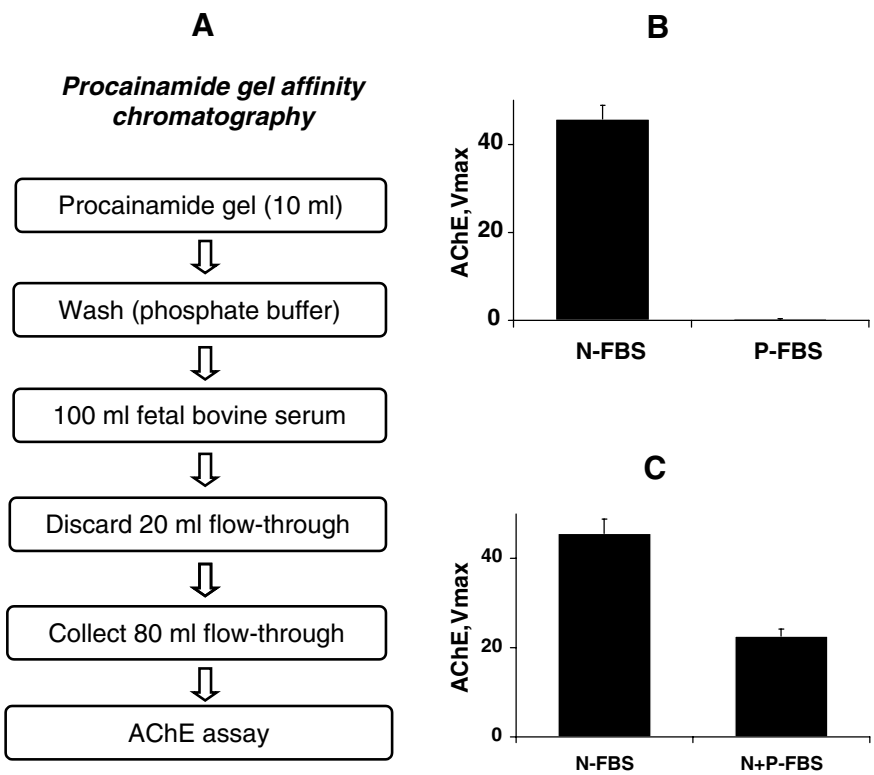

Fig. 1. Depletion of serum cholinesterases using procainamide gel affinity chromatography. **A**: Flow-chart depicting procainamide affinity chromatography of fetal bovine serum (FBS). **B**: Acetylcholinesterase (AChE) enzyme assay shows that procainamide chromatography removes virtually all of the soluble AChE activity present in the serum. Results are expressed as mean ± SEM (n = 4). **C**: Mixing the procainamide depleted serum with untreated serum does not decrease the enzyme more than half showing that procainamide does not leech from the column and therefore will not inhibit the expressed AChE following transcriptional induction.

TSA Upregulates AChE Expression in Neuro-2A Cell Line

Neuro-2A cells (1×10^5 cells/well in 48-well plates) were incubated for 7 days with 10-fold dilutions (0.33, 3.3, 33, 333, and 3,333 nM) of TSA added once at the beginning of the incubation. Microscopy showed that the cell morphology of Neuro-2A was altered in TSA treated cells compared to control vehicle treated cells (Fig. 2A). Production of distinct filamentous projections were observed at 0.33, 3.3, 33, and 333 nM TSA. The neurofilaments started forming networks in cells treated with 33 and 333 nM TSA. TSA was found to be cytotoxic to Neuro-2A cells above 333 nM (Fig. 2A). Most of the cells had undergone apoptosis and appeared round following treatment with 3,333 nM TSA.

The extracellular AChE expression in the culture supernatant and the intracellular AChE were measured as described in "Materials and Methods." In TSA treated cells the extracellular AChE level gradually increased with the dose. The optimum induction of AChE was approximately twofold at 333 nM TSA treatment (Fig. 2B). Since a higher dose of 3,333 nM TSA was cytotoxic to Neuro-2A cells and the level of AChE expression was low. The intracellular AChE level was also dose-dependently increased in Neuro-2A cells when

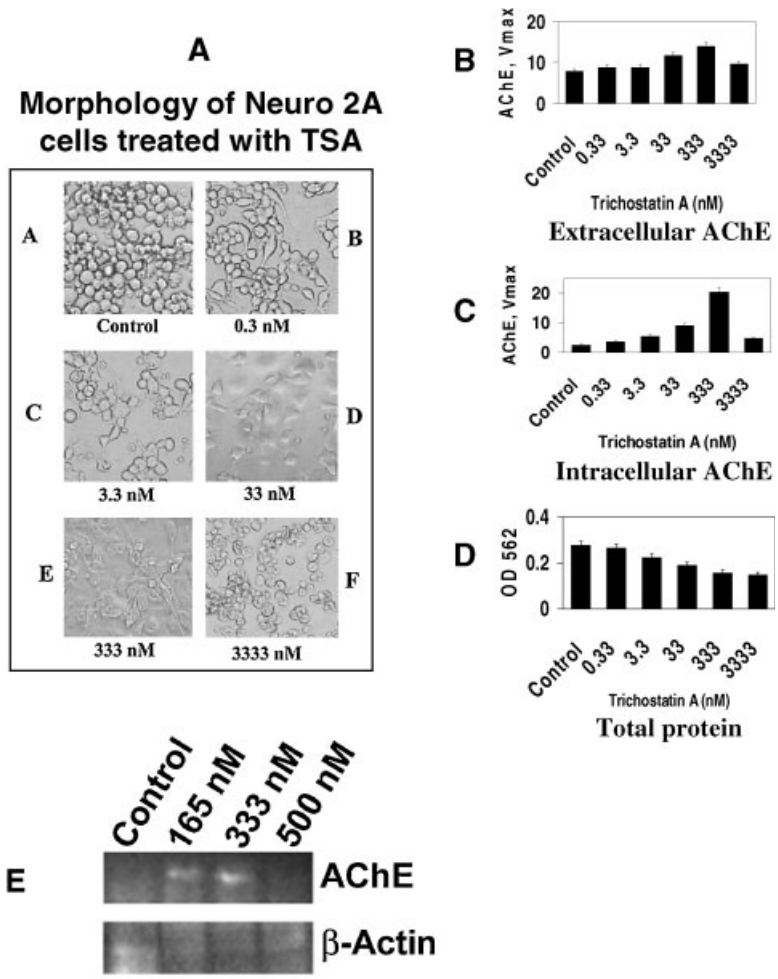

Fig. 2. Effect of trichostatin A (TSA) on Neuro-2A cells. **A**: Photomicrographs (20×) of Neuro-2A control cells or cells treated with various doses of TSA for 7 days. Incubation with TSA produced distinct filamentous projections starting from the dose 0.33–333 nM. **B**: Extracellular AChE activity determined by microassay of 20 µl of the culture supernatant. **C**: Cells were lysed with 1% Nonidet-P40 or T-PER and the intracellular AChE activity determined by using 20 µl of the cell lysate. **D**: Total cellular protein in control Neruo 2A cells and cells treated with various doses of TSA. All the assays were performed in at least triplicates and the variations between repeats were less than 10% of the mean. **E**: RT-PCR using AChE-specific primers indicates increased expression of AChE in 165 and 333 nM TSA treated cells.

treated with increasing concentrations of TSA (Fig. 2C). Consistent with the extracellular AChE level, the highest induction of the intracellular enzyme was also observed in 333 nM TSA treated cells. However, there was approximately an eightfold increase in intracellular expression of AChE compared to untreated controls, while only a twofold was observed extracellularly. Thus, TSA resulted in an accumulation of AChE in the Neuro 2A cells. RT-PCR experiments with AChE primers show that the level of AChE mRNA is increased in cells treated with 165 and 333 nM TSA (Fig. 2E). Further increase in the dose of TSA was cytotoxic and reduced the increased level of AChE mRNA in TSA treated cells. The total cellular protein level was slightly decreased in TSA treated cells at higher dose (Fig. 2D). The decrease in the total protein seemed to be the result of reduced cell proliferation following cell differentiation and neurite outgrowth induced by TSA, especially at higher doses. Further testing of the effect of TSA, using final concentrations (33, 66, 166, 266, and 333 nM) showed that a single treatment with 166 nm TSA induced extracellular and intracellular AChE expression similar to 333 nM TSA treated Neuro-2A cells (data not shown).

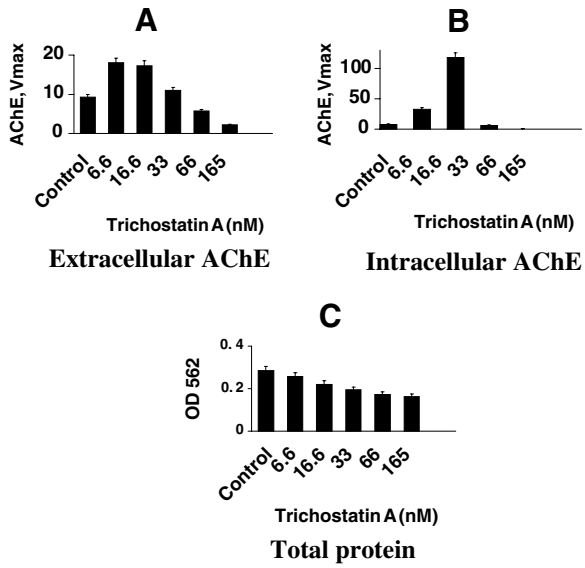

Fig. 3. Effect of repeated addition of TSA on Neuro-2A cells. **A**: Neuro-2A cells were treated repeatedly with various doses of TSA every other day for 7 days. Extracellular AChE activity was determined by microassay using 20 µl of the culture supernatant. **B**: Cells were lysed with 1% Nonidet-P40 and the intracellular AChE activity determined using 20 µl of the cell lysate. Note that the dose of TSA required to induce optimal expression of AChE was significantly reduced when TSA was added repeatedly. **C**: Total cellular protein in Neuro 2A cells repeatedly treated with various doses of TSA. All the assays were performed in at least triplicates and the variations between replicates were less than 10% of the mean.

Treatment of TSA Every Other Day for 7 Days Induces AChE Expression at Lower Doses

To investigate whether multiple doses of TSA added over a 7 day period would induce higher levels of AChE expression, Neuro-2A cells were treated with every second day for 7 days. During the repeated addition, the medium was completely removed and replaced with fresh medium containing TSA. Under these conditions the dose range of TSA that induced AChE maximally was 6.6–16.6 nM (Fig. 3A,B). The extracellular AChE was increased approximately twofold at 6.6 and 16.6 nM TSA. However, the expression of intracellular AChE following repeated TSA treatment was approximately 14-fold higher, compared to controls. Thus, multiple additions of TSA induce higher levels of intracellular AChE compared to single addition. The lower level of increase in the expression of extracellular level of AChE following multiple additions compared to single addition could be due to the changing of the medium during multiple treatments that prevents the accumulation of secreted enzyme. These results indicate that repeated addition of TSA is more effective in inducing AChE expression at lower doses compared to single treatment. The total protein was slightly reduced at TSA doses above the optimum dose that induce AChE expression following treatment of Neuro 2A cells with multiple doses of TSA (Fig. 3C).

Cytochemical Staining for AChE Indicates Increased Expression of the Enzyme Following TSA Treatment

Neuro-2A cells repeatedly treated with TSA and controls were stained with Karnovsky staining as described in "Materials and Methods." Microscopy imaging showed that the staining was very dark in cells treated with 6.6 and 16.6 nM TSA compared to control cells treated with the vehicle (Fig. 4). Strong staining indicates a high level of AChE activity. Increased cytochemical staining of AChE was also demonstrated using a new fluorescent staining techniques using F-150 maelimide (Molecular Probes, Carlsbad, CA) which react

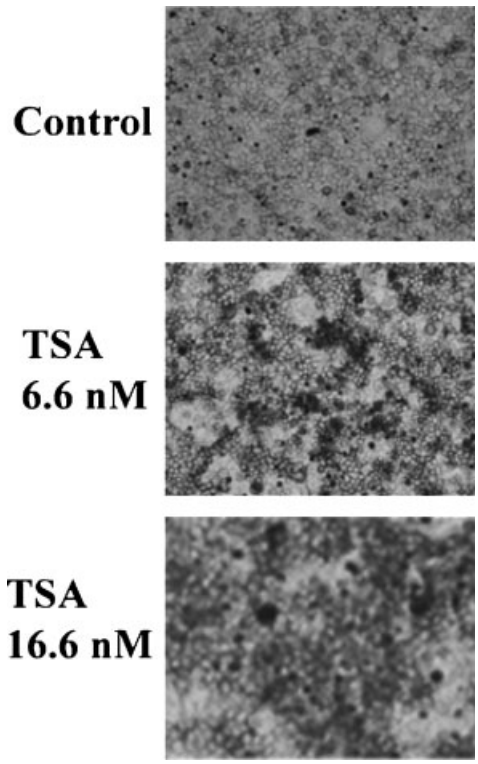

Fig. 4. Cytochemical staining of AChE in TSA treated Neuro-2A cells. Neuro-2A cells were treated with various doses of TSA for 7 days as described in Figure 3. Cytochemical staining by the Karnovsky method was performed as described in the ''Materials and Methods.'' The stained cells were observed under an Olympus microscope at 20×. In consistent with the increased expression of AChE, the staining was significantly increased in TSA treated cells.

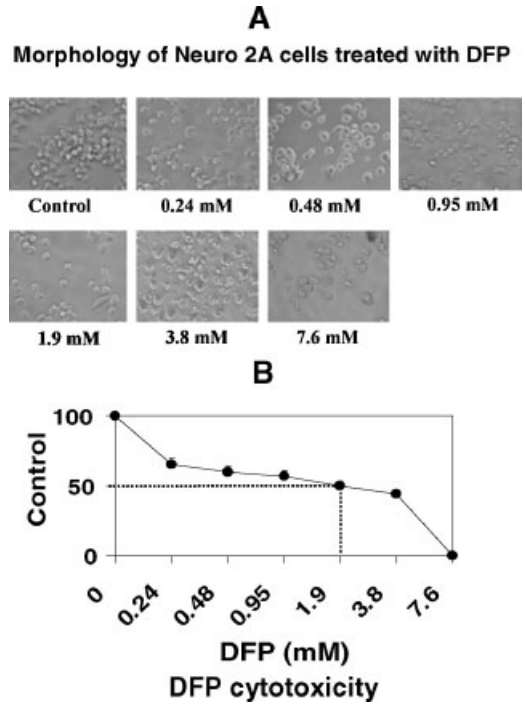

Fig. 5. Effect of diisopropylfluorophosphate on Neuro-2A cells. **A**: Photomicrographs (20×) of Neuro-2A cells treated with various doses of DFP for 3 days. **B**: The cytotoxicity of DFP was measured by the MTT assay as described in ''Materials and Methods'' and represented as percent of control. Extracellular AChE activity was determined by microassay of 20 µl of culture supernatant. All the enzyme assays were performed in triplicates and the variations between replicates were less than 10% of the mean.

with free SH groups that makes it fluorescent (data not shown). These results further confirm that TSA induces the intracellular expression of AChE.

Effect of the Organophosphate Diisopropylfluorophosphate on Neuro-2A Cells

To study the protection afforded by TSA treatment to the cytotoxic effects of organophosphate exposure, we evaluated diisopropylfluorophosphate, a surrogate for the nerve agents soman and sarin, on Neuro-2A cells. The cells (1×10^5 cells/well in 48-well plates) were incubated with 0.24, 0.48, 0.95, 1.9, 3.8, and 7.6 mM DFP for 3 days. DFP-induced cytotoxicity was visible by rounding, floating of dead cells, and reduction in the number of remaining cells. The cell morphology showed that significant cytotoxicity was evident even at the lowest dose of DFP (0.24 mM) (Fig. 5A). The MTT cytotoxicity assay yielded an LD_{50} of 1.9 mM for DFP in Neuro-2A cells (Fig. 5B). Although the cytotoxicity was obvious at lower doses of DFP, a relatively higher dose of DFP was required to kills the cells.

TSA Treatment Protects the Cells From Organophosphate (DFP) Induced Neurotoxicity

To determine whether the increase in the expression of AChE following TSA treatment would protect the cells against OP exposure, Neuro-2A cells (1×10^5 cells/well in 48-well plates) were incubated with 33, 66, 166, and 333 nM TSA for 7 days. Next, DFP (7 mM) was added and the cells were incubated for an additional 3 days. Microscopy results showed that the cells treated first with 33, 66, and 166 nM TSA followed by DFP exposure survived and retained their original cellular morphology (Fig. 6A). Cell survivability was greatest at 166 nM TSA. The protection of cells by TSA

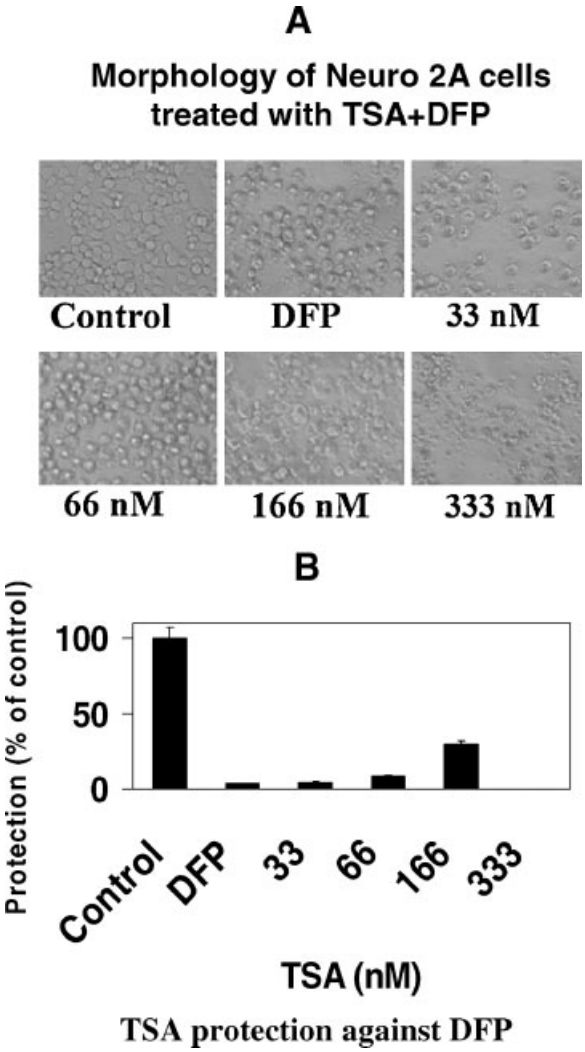

Fig. 6. TSA protects Neuro-2A cells from the cytotoxicity of DFP. **A**: Photomicrographs (20×) of Neuro-2A cells treated with the indicated doses of TSA and then incubated with 7 mM DFP for 3 days. **B**: The protective effect of TSA was measured in DFP treated cells by the MTT cytotoxicity assay as described in ''Materials and Methods.'' The protection was expressed as a percentage of control cells not treated with TSA and DFP. All the assays were performed in triplicates. The variations between replicates were less than 10% of the mean.

against DFP cytotoxicity was measured by the MTT cytotoxicity assay. The protection of the cytotoxicity in TSA and DFP treated cells was expressed as percentage of control samples treated with TSA. Consistent with the cellular morphology, the cytotoxicity data shows that 166 nM TSA significantly protected the Neuro-2A cells against DFP exposure (Fig. 6B). These data suggests that induction of cellular AChE by TSA can bioscavenge the organophosphate (DFP) and reduce its cytotoxicity.

DISCUSSION

We demonstrated in this study that a nerve agent bioscavenger, AChE, can be induced in Neuro-2A cells using the transcriptional enhancer TSA. Transcriptional induction of AChE has been studied in various systems [Fernandes et al., 1998; Wan et al., 2000b]. Nerve growth factor induces AChE 3.5-fold following 72 h in PC 12 cell line [Deschenes-Furry et al., 2003]. Mechanical stimulation increases the expression of AChE in cultured myotubes [Hubatsch and Jasmin, 1997]. Calcitonin gene related peptide significantly induce AChE synthesis in cultured myotubes by a cAMP mediated pathway [Choi et al., 1997, 1998, 2001]. Recently it has been shown that pyridoacridine alkaloids induce a fourfold increase in AChE expression after 48 h [Aoki et al., 2003]. Significant increase in the expression of AChE was also found in mouse neuronal and astrocytes after treatment with β-amyloid peptide. Although several biochemicals induce the expression of AChE, the induction of endogenous AChE for protection against OP exposure has never been tested. We have provided evidence for the first time that the transcriptionally induced AChE was able to protect the cells against the cytotoxicity of a surrogate chemical warfare nerve agent, DFP. These results indicate that transcriptional inducers of AChE could be used as novel pre-treatments for protection against CWNAs.

To accurately measure the induced level of AChE activity, we have depleted the FBS of AChE/BChE using procainamide gel affinity chromatography. Initial experiments showed the presence of high background AChE activity in normal FBS that interfered with the estimation of induced AChE. Procainamide gel completely removed the background serum cholinesterases and reduced the background enzyme levels. FBS is unique in having a soluble form of AChE [Doctor et al., 1989]. However, protein estimation showed very little difference in the level of protein before and after procainamide gel electrophoresis suggesting that very few proteins are removed non-specifically by the procainamide gel. Accordingly, cell growth was found be normal medium containing procainamide treated serum compared to untreated serum.

Our data indicates that TSA induces the expression of both intracellular and extracellular AChE expression. Although the mechanism

of induction involves de-condensation of the chromatin and increased binding of the transcription factors, the exact nature of the transcription factors it is not known. It has been shown that AChE promoter has a GC-rich region −105 to −59 base pairs from the start of transcription containing overlapping sites for the transcription factors Sp1 and Egr-1 is essential for promoter activity [Mutero et al., 1995]. Histone acetylase inhibitors augment Sp1 acetylation and Sp1 DNA binding and Sp1 dependent gene expression [Ryu et al., 2003]. Intronic N- and E box motifs contribute for the myogenin-induced increase in AChE and play a role in the developmental regulation of the enzyme [Angus et al., 2001]. However, it seems that the synthesis of AChE is slow following incubation with TSA. Investigation of AChE expression on the first or second day after the addition of TSA showed a small increase in the expression of AChE. The AChE expression was gradually increased when the cells were incubated for a longer time.

High levels of induction of intracellular AChE compared to extracellular AChE levels indicate that significant amount of the enzyme is accumulated in the cells. This could be possibly due to the difference in the ability of the cells to release the enzyme to the culture medium. Differential cellular localization and secretion of the enzyme in cell lines has been reported previously [Inestrosa et al., 1981, 1985; Melone et al., 1987]. From the perspective of protection against CWNAs, the increased levels of intracellular AChE have an important advantage over externally administered enzymes. The administered enzyme is unable to get into the cells and cannot protect against the cellular toxicity of OPs. On the other hand transcriptionally induced increase in the levels of intracellular enzyme can scavenge the endocytosed OPs. It has been shown that OPs have important non-AChE targets in the cells including other serine proteases and neuropathy target esterase's [Duysen et al., 2001; Casida and Quistad, 2004]. Inhibition of neuropathy target esterase leads to the organophosphate induced delayed neuropathy (OPIDN) [Funk et al., 1994; Roldan and Sanchez, 2004].

Increased induction of AChE and reduction in the optimal dose following repeated treatment with TSA indicate that a continuous presence of the drug is required for maximal induction. This is possibly due to the metabolism and degradation of TSA that could result in the reversal of AChE induction. Our previous studies with TSA indicate that the drug is degraded within 24 h of treatment in human lymphocytes [Nambiar et al., 2002]. Repeated addition also shows a cumulative effect in the induction of intracellular AChE. Following repeated treatment, intracellular AChE was increased more than single time treatment with TSA. Thus a constant pressure of the transcription factor is required for sustained expression of intracellular AChE.

The approach of using transcriptional inducers for prophylactic therapy of chemical warfare nerve agent exposure has several advantages. Currently, purified FBS AChE or human plasma BChE is being used as a pretreatment for OP exposure. Therapy involving induction of self-human plasma BChE will minimize the production of antibodies formed due to the complex structure and posttranslational modification of the parentally administered purified cholinesterases derived from pooled human plasma. Also, the cost of drug, labor, and treatment will be much lower by using transcriptional inducers compared to purified enzyme treatments that need a large amount of human plasma. Another advantage of using transcriptional inducers is that the enzyme level can be maintained high as long as the inducer is present in the body and will not need invasive methods of administration. Moreover, induction of intracellular AChE can protect against the intracellular toxicity of OPs, unlike the administration of purified enzyme that does not endocytose to the cells. On a separate note, transcriptional inducers can also be used to increase the synthesis of native or recombinant of AChE to improve the yield of purified enzyme. In our cellular model, experimenting with perhaps a purified enzyme plus TSA treatment would be somewhat fallible, due to the artificially high protection that thepurified enzyme would give. In cell culture the half life of the purified enzyme could be longer and would synthetically protect the cells better.

It is known that several FDA-approved drugs that currently in use cause a certain type of lupus known as "drug-induced lupus." Although the authors previously reported that TSA at high doses causes abnormalities in T cell signaling, it could be due to the drug-induced lupus effect. TSA itself has been reported to

protect against lupus [Mishra et al., 2001] and is in the process of being submission for FDA approval. Therefore, the drug may be used to induce AChE production for OP treatment while the other effects remain minimal or a low dose of the drug can be used for long-term effects.

Currently, drugs based on transcriptional regulation are in their infancy. Transcriptional inducers are being seriously investigated in the treatment of many disorders that could be ameliorated by differential gene expression. TSA is currently being considered for use as an anti-tumor agent [Vanhaecke et al., 2004]. In the studies mentioned, the negative affects of TSA on cytotoxicity were shown to be minimal. Thus, our study supports a pre-treatment option for organophosphate exposure by transcriptional inducers and opens a new avenue for the protection against chemical warfare agents. There is, however, some evidence that suggests that TSA could have an influence on the expression of other proteins that could have negative effects. For example, TSA induces the expression of the prion protein [Cabral et al., 2002]. The mutation of the prion protein has been linked to severe neurodegenerative diseases [Castilla et al., 2004]. Prion-related diseases require the expression of the prion proteins, and therefore an up-regulation of the prion protein has to be considered a risk factor for TSA treatment under clinical settings.

In summary, we show that the activity of the chemical warfare nerve agent bioscavenger AChE can be induced by the transcriptional inducer TSA. The induced enzyme can bioscavenge the organophosphate (DFP), thereby protecting the cells from OP-induced cytotoxicity. Thus, transcriptional inducers of AChE could be used as novel antidotes for the treatment of chemical warfare agents which are potent inhibitors of cholinesterases.

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