EFFECTS OF THIMEROSAL ON NGF SIGNAL TRANSDUCTION AND
CELL DEATH IN NEUROBLASTOMA CELLS

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ABSTRACT

Signaling through neurotrophic receptors is necessary for differentiation and survival of the developing nervous system. The present study examined the effects of the organic mercury compound thimerosal on nerve growth factor signal transduction and cell death in a human neuroblastoma cell line (SH-SY5Y cells). Following exposure to 100 ng/ml NGF and increasing concentrations of thimerosal (1 nM – 10 µM), we measured the activation of TrkA, MAPK and PKC-δ. In controls, the activation of TrkA MAPK and PKC-δ peaked after 5 minutes of exposure to NGF and then decreased but was still detectable at 60 minutes. Concurrent exposure to increasing concentrations of thimerosal and NGF for 5 minutes resulted in a concentration-dependent decrease in TrkA and MAPK phosphorylation, which was evident at 50 nM for TrkA and 100 nM for MAPK. Cell viability was assessed by the LDH assay. Following 24 hr exposure to increasing concentrations of thimerosal, the EC₅₀ for cell death in the presence or absence of NGF was 596 nM and 38.7 nM, respectively. Following 48 hr exposure to increasing concentrations of thimerosal, the EC₅₀ for cell death in the presence and absence of NGF was 105 nM and 4.35 nM, respectively. This suggests that NGF provides protection against thimerosal cytotoxicity. To determine if apoptotic versus necrotic cell death was occurring, oligonucleosomal fragmented DNA was quantified by ELISA. Control levels of fragmented DNA were similar in both the presence and absence of NGF. With and without NGF, thimerosal caused elevated levels of fragmented DNA appearing at 0.01 µM (apoptosis) to decrease at concentrations > 1 µM (necrosis). These data demonstrate that thimerosal could alter NGF-induced signaling in neurotrophin-treated cells at concentrations lower than those responsible for cell death.

Keywords: signal transduction, neurotrophin, mercury compound
INTRODUCTION

Thimerosal is an organic mercury compound that is used as a preservative in many vaccines due to its antibacterial and antifungal abilities. It consists of an organic radical, ethylmercury (49.6% by weight), which is bound to the sulfur atom of the thiol group of salicylic acid. The type of anion attached to ethylmercury affects neither the distribution of mercury in the body nor its toxicity (Suzuki et al. 1973; Ulfvarson 1962), while the organic radical has a strong impact on both (Magos 2003). Ethylmercury and its decomposition product, Hg^{2+}, rapidly accumulate in the tissues (Magos 2001), preferentially in the kidneys and brain (Blair et al. 1975). Following in vivo administration, ethylmercury passes through cellular membranes and concentrates in cells of vital organs, including the brain, where it releases inorganic mercury, raising its concentrations higher than equimolar doses of its close and highly toxic relative methylmercury (Magos et al. 1985).

There has recently been concern about the effects of this source of mercury on the fetal and infant nervous system, especially in infants who develop neurodevelopmental disorders such as autism, attention deficit-hyperactive disorder (ADHD) and speech or language delay (Bernard et al. 2001; Kidd 2002). Human studies do not show a significant association between the use of thimerosal-containing vaccines and the development of autism in children. More studies on this topic need to be completed to obtain conclusive results (NAS, 2001).

Some recent in vitro studies show that certain concentrations of thimerosal have decreased cellular viability in human neurons and fibroblasts. For example, Baskin et al. (2003) noted an increase in membrane permeability to DAPI dye as early as 2 hours after incubation of human cortical neurons and fibroblasts with 250 µM thimerosal. A 6 hour incubation resulted in
membrane damage (loss of DAPI dye exclusion), DNA breaks, and apoptosis as indicated by morphology and caspase-3 activation (Baskin et al. 2003; Makani et al. 2002).

The studies cited above identified a number of molecular targets for thimerosal, including micronuclei induction, disturbances of intracellular calcium, and inhibition of glutathione content (Ueha-Ishibashi et al., 2005a; 2004b; Westphal et al., 2003), but the unique dependence of the developing nervous system on growth factors suggests that the neurotrophins and their receptors represent a possible target for thimerosal. There are several studies suggesting that thimerosal may alter neurotrophin signaling, including binding of secondary messengers (Vanlingen et al. 2001), microtubule assembly (Alexandre et al. 2003), and intracellular calcium concentrations (Ueha-Ishibashi et al. 2004a). Concentrations of thimerosal showing an effect on the development and viability of undifferentiated human neuroblastoma cells and neurotrophin cell signaling in terms of protein phosphorylation and cell viability are of interest.

Neurotrophins, especially nerve growth factor (NGF), but also the related neurotrophins, brain-derived growth factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), are known to affect the biological behavior of neuronal cells during development, survival and differentiation of the central and peripheral nervous systems (Barde 1989; Thoenen 1991). The role of NGF in thimerosal-induced cytotoxicity has not been determined. In the present study, the SH-SY5Y human neuroblastoma cell line (SY5Y cells) was used as an in vitro model for neurotrophin (NGF)-induced differentiation. SY5Y cells represent a well characterized in vitro model system used to study the actions of neurotrophins on developing neurons (Eggert et al. 2001; Soderholm et al. 2001). In addition, continuously dividing cell lines such as SY5Y cells have been widely used as a model to study neurodegeneration (Peraus et al. 1997; Sheehan et al.)
SY5Y cells differentiate into cells that are biochemically, ultrastructurally and electrophysiologically similar to neurons (Abemayor and Sidell 1989). In this cell line, activation of the TrkA receptor by its ligand NGF leads to differentiation (Nakagawara et al. 1993). These actions of NGF on SY5Y cells are dependent on the high affinity interaction with the TrkA receptor (Martin-Zanca et al. 1989). Binding of NGF to the TrkA receptor induces receptor dimerization and tyrosine kinase activity that results in autophosphorylation of several tyrosine residues on the cytoplasmic domain of the receptor (Kaplan et al. 1991). These phosphorylated tyrosines serve as anchors for binding and activating downstream signaling elements such as phospholipase $C_\gamma$ (PLC), Shc, SNT and phosphatidylinositol 3-kinase (Greene and Kaplan 1995; Kaplan and Miller 1997). These proteins couple TrkA to several intracellular pathways such as the mitogen-activated protein kinases (MAPK) cascade and protein kinase C (PKC) (Greene and Kaplan 1995). Activation of the MAPK cascade by NGF leads to activation of transcription factors and induction of immediate-early genes, ultimately leading to differentiation (i.e., neurite outgrowth) (Greene and Kaplan 1995; Kaplan and Miller 1997). Specific isoforms of PKC have been associated with NGF-induced differentiation, in particular PKC-δ and PKC-ε (Brodie et al., 1999; Corbit et al., 1999). PKC-δ differs in that it is translocated to the membrane in response to NGF (O’Driscoll et al., 1995). In addition, PKC-δ is required for NGF-induced activation of the MAPK cascade, and contributes to MEK-induced neurite outgrowth (differentiation) (Corbit et al., 1999). In the present study, we examined the NGF signal transduction cascade in human neuroblastoma SY5Y cells that were stimulated with NGF in the presence of various concentrations of thimerosal. The effects of thimerosal on NGF-induced TrkA autophosphorylation, MAPK activation and PKC-δ phosphorylation were determined. Our data support the hypothesis that NGF signaling through TrkA is altered with
subsequent inhibition of signaling through the MAPK cascade upon thimerosal exposure at low, non-cytotoxic micromolar concentrations.

MATERIALS AND METHODS

Materials. SH-SY5Y cells were obtained from the American Type Culture Collection (Rockville, MD). Human recombinant nerve growth factor-beta (NGF), rat-tail collagen type I and thimerosal were purchased from Sigma Chemical Company (St. Louis, MO). Minimum essential medium (MEM), MEM non-essential amino acids solution, Ham’s F-12 medium, Trypan Blue Solution and fetal bovine serum (FBS) were purchased from Life Technologies, Rockville, MD). Six- and 96-well plates, 100 x 20- mm culture dishes, and 75 cm² flasks were purchased from Corning (Corning, NY).

Cell Culture. SY5Y cells were routinely seeded at 5 x 10⁴ – 1 x 10⁵ cells/ml in collagen-coated 75 cm² tissue culture flasks. The cells were grown in 1:1 MEM and Ham’s F-12 medium containing 10% FBS and 10% MEM non-essential amino acids solution (SY5Y medium). Cultures were maintained according to standard protocols at 37°C in a 95% humidified incubator with 5% CO₂. SY5Y cell medium was replaced every 2-3 days until cells reached >70% confluency, at which time they were harvested or plated for incubations with NGF (100 ng/ml) and/or thimerosal in the assays described below. Thimerosal was diluted from a 0.1 M stock solution prepared in sterile distilled water. Final exposure concentrations of 1 nM to 10 µM were obtained by 1:100 dilutions. For experiments with thimerosal, control wells were dosed with an equivalent volume of the vehicle (sterile, distilled water), which did not cause any adverse effects.

Western Blot Analysis. After harvest, cells were washed three times with ice-cold
phosphate-buffered saline (PBS). SY5Y cells were then collected by the addition of 0.5 ml lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM NaF, 1 mM Na3VO4, 1:200 dilution of Protease Inhibitor Cocktail Set III (Calbiochem, La Jolla, CA)] and gentle rocking at 4°C for 20 min. The lysed cells were then centrifuged at 10,000g for 10 min at 4°C. An aliquot of the supernatant was taken for protein determination using a commercially available kit (Pierce, Rockford, IL) based on the bicinchoninic acid (BCA) method. The remaining supernatant was added to an equal volume of Laemmli sample buffer (62.5 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol), heated to 95°C for 5 min, and stored at –80°C.

Duplicate aliquots of cell lysates in sample buffer (15-30 µg protein) were subjected to SDS-PAGE using 7.5 or 10% polyacrylamide gels. Gels were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) in 48 mM Tris, pH 6.8, 39 mM glycine, 0.00375% SDS, and 20% methanol. Membranes were washed in tris-buffered saline (TBS, 50 mM Tris, 0.9% NaCl, pH 7.5) and blocked for 1 h in TBS containing 5% non-fat dry milk and 0.03% Tween-20. The membranes were then incubated overnight at 4°C with a commercially available rabbit polyclonal primary antisera recognizing the following protein epitopes: TrkA (Santa Cruz, Santa Cruz, CA), TrkB (Santa Cruz), TrkC (Santa Cruz), PLCγ (Upstate Biotechnology, Charlottesville, VA), PKC-δ (Cell Signaling, Beverly, MA), PKC-ε (Cell Signaling), cyclic AMP response element binding protein (CREB, Cell Signaling), protein kinase B (AKT, Cell Signaling), mitogen activated protein kinase (MAPK, Cell Signaling) and phosphorylation state-specific antibodies that recognize the activated form of TrkA (Tyr496; Santa Cruz), MAPK (Thr2002 / Tyr204; Cell Signaling) or PKC-δ (Tyr311; Cell Signaling).

Membranes were washed again in TBS and subsequently incubated for 1 hour at room
temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000; Santa Cruz). Detection was performed using an enhanced chemiluminescent system (Pierce, Rockford, IL). Densitometry analysis was performed using the NIH Image 1.63 software, with n = 3 - 4 blots.

**Lactate Dehydrogenase (LDH) Assays.** SY5Y cells were plated in 96-well plates at 2.5 x 10^4 cells per well in 200 µl/well SY5Y medium. After 24 hr, the SY5Y medium was removed and replaced with serum-free SY5Y medium in the presence or absence of 100 ng/ml NGF and increasing concentrations of thimerosal (1 nM – 10 µM). Following 24 hr exposure, 100 µl/well of medium was carefully removed from each well and transferred into corresponding wells of an optically clear 96-well flat bottom microtiter plate. To determine LDH activity in this medium, the LDH Cytotoxicity Detection Kit was used (Roche, Indianapolis, IN), with 100 µl of the reaction mixture added to each well. Following a 20 min incubation at room temperature and protection from light, the absorbance was measured at 492 nm (reference wavelength 600 nm) on a microplate spectrophotometer, with n = 3 samples.

**DNA Fragmentation ELISA.** SY5Y cells were plated in 6-well plates at 1 x 10^5 cells per well in 2 ml SY5Y medium. After 24 hr, the SY5Y medium was removed and replaced with serum-free SY5Y medium in the presence or absence of 100 ng/ml NGF and increasing concentrations of thimerosal (1 nM – 10 µM). Following 24 hr exposure, the SY5Y cells were removed from the cell culture plates and placed in a 2 ml centrifuge. The cell suspension was centrifuged at ~200 g for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in serum-free medium. The cell suspension was centrifuged at ~200 g for another 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in 500 µl of incubation buffer (Cell Death Detection ELISA, Roche). The cell suspension was incubated in the incubation buffer for 30 min at room temperature, followed by
centrifugation at ~20,000 g for 10 min. Four hundred microliters of supernatant were removed and 25 µl were diluted 1:10 with incubation buffer. Microplates were coated with an anti-histone antibody and washed with washing solution and incubation buffer before the cell homogenate sample was added. The cell homogenate was incubated in the anti-histone coated plates for 90 min at room temperature and washed three times with the wash solution. An anti-DNA-POD antibody was incubated with the samples for 90 min at room temperature and washed three times with the wash solution. DNA fragmentation was determined by adding the substrate solution and absorbance was measured at 405 nm.

**Statistics.** Curves were fit to concentration-response data using non-linear regression analysis (GraphPad Software, San Diego, CA). Data were analyzed for EC$_{50}$ values and statistical significance by one-way analysis of variance (ANOVA) using Instat Software (GraphPad Software). In the case of a significant ANOVA, post-hoc analysis was performed using Tukey’s test (or Dunnett’s test when comparisons were to control). The level of significance was set at p<0.05.

**RESULTS**

**Characterization of the SY5Y Cell Model.** All but TrkC of the proteins tested were expressed in SY5Y cells. Therefore, protein expression of the following NGF-induced signal transducing proteins was observed in our SY5Y cell model: TrkA, TrkB, TrkC, MAPK, PLC$_{γ}$, PKC-δ, PKC-ε, CREB and AKT (Fig. 1). Following 30 minutes of exposure to NGF, TrkA autophosphorylation was examined in SY5Y cells and the phosphorylation state-specific TrkA antibody detected a single band at 140 kD that corresponded to activated TrkA with maximal protein phosphorylation (Fig. 2A). A second antibody, which recognizes TrkA regardless of the
phosphorylation state, also detected a single band of protein at 140 kD in both control and NGF-treated cells. NGF-induced activation (phosphorylation) of MAPK, the last step of the MAPK cascade, was determined at 0, 5, 15, 30 or 60 minutes (Fig. 2B). Both the phospho-specific and total MAPK antibodies were detected as bands at 42 and 44 kD. We observed a sustained activation of MAPK that peaked at 5 min of NGF exposure, but was detectable for up to 60 min (Fig. 2B). NGF-induced phosphorylation of PKC-δ was also determined to be altered by time after exposure to NGF, with peak protein phosphorylation following 5 min of NGF exposure. It decreased but protein phosphorylation was still detectable for up to 60 min (Fig. 2C).

Effects of Thimerosal on NGF-induced activation of TrkA, MAPK, PKC-δ. We determined the effects of thimerosal on NGF-induced TrkA, MAPK and PKC-δ activation. For these experiments, SY5Y cells were exposed to increasing concentrations of thimerosal and 100 ng/ml NGF for 5 min, which was the peak time of NGF-induced activation. Cells were harvested and the activation state was determined using phosphorylation state-specific antibodies that only recognize the activated form of TrkA, MAPK and PKC-δ (Fig. 3). Following 5 min exposure to NGF and increasing concentrations of thimerosal, there was a concentration-dependent decrease in TrkA autophosphorylation (Fig. 3A) and MAPK phosphorylation (Fig. 3B), with EC50 concentrations of 49.8 ± 7.5 and 57.5 ± 0.28, respectively. Effects on PKC-δ were less notable and did not reach statistical significance (Fig. 3C).

Effects of Thimerosal on Overt Cell Death. To determine the effects of thimerosal on cell death, SY5Y cells were exposed for 24 or 48 hr to increasing concentrations of thimerosal in the presence and absence of NGF and cell death was examined by measuring LDH activity. Following exposure to thimerosal for 24 hr, there was a concentration-dependent decrease in cell viability either in the absence or presence of NGF (Fig. 4). The EC50 for cytotoxicity was lower.
in the absence of NGF (38.7 ± 2.57 nM; mean ± SD, n = 3) than in the presence of NGF (596 ± 11.42 nM). Following exposure to thimerosal for 48 hr, there was also a concentration-dependent decrease in cell viability either in the absence or presence of NGF (Fig. 4), with EC$_{50}$’s of 4.35 ± 0.791 nM and 105 ± 11.0 nM, respectively. All EC$_{50}$ values were significantly different from one another. In total, the cell viability data indicate that thimerosal cytotoxicity was dependent both on the concentration of thimerosal and the length of time the cells were exposed to this organic mercury compound.

The effects of thimerosal on DNA fragmentation were assessed with the ELISA assay. In control SY5Y cells, there was no difference in fragmented DNA in the absence or presence of NGF (raw values not shown). Following 24 hour exposure to increasing concentrations of thimerosal in the absence of NGF, fragmented DNA increased above control values, with highest values at 0.01 µM (to 157%, 0.05 < p < 0.1). An increase in DNA fragmentation over controls was also seen at this concentration of thimerosal in cell incubates containing NGF (121%, p=0.04). With or without NGF, there was a significant decrease in fragmented DNA as thimerosal concentrations increased from 0.01 µM to 1 µM (p<0.05) (Fig. 5).

**DISCUSSION**

In the present study, we used an *in vitro* culture system as a model to examine the effects of thimerosal on neurotrophin signaling and survival in SY5Y cells. These cells serve as a model for investigating biomolecular events involved in neuronal differentiation. SY5Y cells expressing TrkA respond to NGF by undergoing differentiation *in vitro* (Gryz and Meakin 2003). Previous examination of TrkA signaling in SY5Y cells has indicated that NGF-dependent differentiation of SY5Y cells depends on TrkA kinase activity (Eggert *et al.* 2001). Initially,
protein expression of the neurotrophin receptors was characterized in the SY5Y cells. In SY5Y cells that have not previously been exposed to exogenous neurotrophins, there is high expression of TrkA and moderate expression of TrkB, with no expression of TrkC. High expression of TrkA can be expected in cells from a neuroblastomas cell line (Nakagawara et al. 1993). While high expression of TrkC has been reported with medulloblastomas (Segal et al. 1994). NGF binding to TrkA induces receptor dimerization and autophosphorylation (Loeb and Greene 1993; Stephens et al. 1994). In the present study, we used the SY5Y cell model to examine the effects of thimerosal on the initial (TrkA receptor autophosphorylation) and downstream (MAPK and PKC-δ) events of NGF signaling. We tested expression of NGF-stimulated cell signaling proteins in the presence of various concentrations of thimerosal. The present study used a phospho-specific antibody to examine thimerosal effects on autophosphorylation of tyrosine 490 (tyr 490) of TrkA. Under control conditions, autophosphorylation of tyr 490 occurred rapidly upon exposure to NGF. Peak activity was observed at 5 min, the earliest time-point examined. Autophosphorylation then decreased but remained above control levels for the next 60 min. These observations agree with earlier findings in both undifferentiated (proliferating) (Kaplan et al. 1991; Qiu and Green 1991) and differentiated (Zhou et al. 1995) PC12 cells.

Concurrent exposure to NGF and thimerosal decreased TrkA autophosphorylation. The inhibition of TrkA autophosphorylation was concentration-dependent and was evident at thimerosal concentrations as low as 50 nM. Inhibition of TrkA activity is likely to have consequences on downstream signaling. As described above, TrkA activation leads to the initiation of several effects including Rac1, the MAPK cascade, PLCγ, and PKC (Martin-Zanca et al. 1989; Szeberenyi and Erhardt 1994). The timecourse for activation of these proteins follows closely to that of TrkA (Kao et al. 2001; Zhou et al. 1995). Studies have demonstrated that
another neural cell line (PC12 cells) can either differentiate or proliferate in response to growth factor stimulation according to the strength (threshold) or duration (or both) of the stimulus (Dikic et al. 1994; Marshall 1995). The mechanism is not clear, but it has been shown that both the strength and duration of the signal generated by a receptor with tyrosine kinase activity can influence the downstream signaling pathway, leading to cell differentiation instead of cell proliferation (Dikic et al. 1994; Traverse et al. 1992). Therefore, it is possible that thimerosal-induced inhibition of the early peak of neurotrophin signaling may reduce TrkA activity below a threshold, leading to effects on downstream signaling and differentiation in SY5Y cells.

Phosphorylation of tyrosine 490 and 785 within the TrkA receptor leads to activation of the MAPK cascade and PKC, respectively (Fujita et al. 1989). The MAPK cascade has been shown to be important for NGF signaling (Kao et al. 2001; Traverse et al. 1992). The present study used a phospho-specific antibody to examine thimerosal effects on the phosphorylation of MAPK, which is the final step of the MAPK cascade (Stephens et al. 1994). In the absence of thimerosal, phosphorylation of MAPK in SY5Y occurred rapidly upon exposure to NGF and peak activity was observed at 5 min. MAPK phosphorylation then decreased but remained above the initial (0 min) level of phosphorylation for the next 60 min. Concurrent exposure to NGF and thimerosal decreased MAPK phosphorylation. A more detailed examination revealed a concentration-dependent inhibition with effects observed at concentrations of thimerosal as low as 100 nM. The inhibition of MAPK phosphorylation by thimerosal may be a consequence of its actions upstream on TrkA, or due to a direct effect on enzymes in the MAPK cascade. Further studies that examine the direct effects of thimerosal on recombinant MEK, MAPK1 and MAPK 2 could be used to address the later possibility. While these data will not rule out an effect of
thimerosal on other proteins in the MAPK cascade (including Raf and Ras), it would suggest that TrkA signaling through MAPK is a sensitive target for thimerosal.

PKC activation is known to play a role in differentiation and proliferation (Kiley et al. 1996) and NGF can stimulate the PLC\(\gamma\)/PKC pathway in PC12 cells (Corbit et al. 2000). It has been previously reported that induction of differentiation in SY5Y cells is concomitant with a sustained increased activity of PKC (Lavenius et al. 1994; Parrow et al. 1995). SY5Y cells express at least six isoforms of PKC (\(\alpha\), \(\beta\), \(\delta\), \(\epsilon\), \(\zeta\) and \(\mu\)), but of particular interest in this study was the isoform PKC-\(\delta\), which is associated with NGF-induced differentiation (Corbit et al., 1999; O’Driscoll et al., 1995; Fagerstrom et al. 1996; Zeidman et al. 1999). In addition, previous studies have shown that organic mercury can inhibit PKC activity in vitro (Parran et al. 2004; Speizer et al. 1989), including partially purified PKC from mice and rat brain (Inoue et al. 1988). The present data shows that NGF-induced activation of PKC-\(\delta\) was not significantly altered by thimerosal, which contrasts with effects of thimerosal on TrkA and MAPK.

Our data indicate that the thimerosal cytotoxicity to SY5Y cells depended on both the presence of NGF and the time of exposure to thimerosal. Following a 24 hour exposure in the absence of NGF, thimerosal toxicity was approximately 15 times higher when compared to thimerosal exposure in the presence of NGF. This trend was also observed following a 48 hour exposure where thimerosal effects of cell viability was approximately 24 times higher in the absence of NGF. This may be attributed to NGF’s ability to promote survival as well as regulate and modulate differentiation (Jackson et al. 1990) and some investigators have suggested that NGF acts to help stabilize the reorganization of cytoskeletal elements of cells (Heidemann et al. 1985). In the present study, the absence of NGF and serum may cause increased thimerosal-induced cytotoxicity in a manner similar to that reported previously (Rabizadeh et al., 1993).

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When comparing the EC$_{50}$’s of cytotoxicity, thimerosal toxicity was also time dependent. In the presence and absence of NGF, thimerosal cytotoxicity was 6 and 9 times higher, respectively, following a 48 hour exposure compared to a 24 hr exposure. We showed that thimerosal-induced cytotoxicity in SY5Y cells ranged from low nanomolar to the micromolar concentrations. Similar to our results, high cellular toxicity of thimerosal in low micromolar concentrations was reported using other cell culture models including human neurons and fibroblasts (Baskin et al. 2003) and Jurkat cells (Makani et al. 2002). These studies also concluded that thimerosal exposure resulted in caspase-3 activation, which is induced during apoptotic cell death.

Following 24 hours of exposure to thimerosal, DNA fragmentation first increased and then decreased, with fragmentation highest at 0.01 µM and significantly lower at > 1 µM. Although further experiments are needed for verification, the elevated DNA fragmentation could suggest that thimerosal was inducing apoptotic cell death in a concentration-dependent fashion, perhaps through a mechanism similar to that induced by serum / neurotrophin withdrawal (Batistatou and Greene 1993; Pittman et al. 1993). Apoptosis plays an important role during neuronal development and defects in apoptosis may underlie various neurodegenerative disorders. The process of DNA fragmentation into specific oligonucleosomal fragments has been found to accompany apoptosis in many cell types and has become a biochemical hallmark of classic apoptosis (Wyllie 1980). At concentrations above 1 µM of thimerosal, there was a steep decline in DNA fragmentation. This steep decline in DNA fragmentation was observed in both the presence and absence of NGF. This would suggest that at these higher concentrations, cell death was produced through non-apoptotic pathways.
Mercury species, including thimerosal, are reported to have effects on the antioxidant status of various cell types including astrocytes and neurons (James et al., 2005; Sanfeliu et al., 2001), lymphocytes (Ueha-Ishibashi et al., 2004b) and thymocytes (Macho et al., 1997). Previous mechanistic studies of methylmercury toxicity have implicated reactive oxygen species (ROS) and depletion of intracellular glutathione as a contributor to mercury-induced cytotoxicity (Sanfelie et al., 2001). Glutathione provides the major intracellular defense against ROS and oxidative stress-induced cell damage and apoptosis (Meister, 1995). Its depletion was shown to precede the increase in ROS associated with loss of viability and apoptosis (Macho et al., 1997). Previous studies have demonstrated that thimerosal neurotoxicity is associated with glutathione depletion (James et al., 2005; Ueha-Ishibashi et al., 2004b) and this action is likely to be related to 1) changes in cellular redox status modulates channel and receptor activities (Pessah, 2001; Choi and Lipton, 2000) and 2) cell growth and death are related to cellular redox state (Mates et al., 2002; Hampton and Orrenius, 1998).

In the present study, we observed significant inhibition of TrkA autophosphorylation and MAPK activation at 0.1 µM thimerosal. These effective concentrations are at lower concentrations than what was observed in other proposed modes of action for the developmental neurotoxicity of methylmercury, including disruptions of microtubules (0.5 – 2 µM, (Castoldi et al., 2000; Graff et al., 1997; Wasteneys et al., 1988), decreased expression of NCAM (1 – 2 µM, (Dey et al., 1999; Lagunowich and Grunwald, 1991) and inhibition of the cell cycle and induction of Gadd45/153 (2 – 3 µM, (Ou et al., 1997; Ponce et al., 1994). Effects at lower concentrations were observed following exposure to methylmercury in PC12 cells, with significant inhibition of total neurite outgrowth at 0.03 M (Parran et al., 2001); significant
inhibition of TrkA autophosphorylation at 0.1 M methylmercury (Parran et al., 2003), and significant inhibition of MAPK activation at 0.1 – 0.3 M methylmercury (Parran et al., 2004).

The present data do not exclude the possibility that thimerosal can act at other sites, either directly or indirectly, to inhibit NGF-induced signaling. In response to NGF stimulation, a significant fraction of MAPK is redistributed to the nuclei and is retained there for several hours (Gonzalez et al. 1993). This enables transmission of neurotrophin signaling to the nucleus, where an important end result is transcriptional control. Nuclear uptake is strongly correlated with MAPK-dependent regulation of DNA synthesis in differentiating PC12 cells (Traverse et al. 1992) and appears to require positive signaling through the MAPK cascade and phosphorylation of MAPK (Pang et al. 1995). Several proteins have been identified as putative substrates for MAPK (reviewed by Szeberenyi and Erhardt 1994), including pp90^rsk, PLA2, p62^TCF, c-Fos and c-Jun. Thimerosal could affect these pathways indirectly by inhibiting MAPK activation or directly by inhibiting one of the components of the cascade. In light of the proclivity of thimerosal to bind any protein containing sulfhydryl groups, it is likely that the effects of thimerosal on differentiation are the result of multiple sites of action. A comparison of the effective concentration at these various sites will be necessary to determine the critical actions of thimerosal, which ultimately result in the disruption of differentiation.
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PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. 


LEGENDS

Fig. 1: Neurotrophin Signaling Protein Expression in SY5Y cells. Whole-cell extracts (30 mg total protein per lane) were resolved by SDS-PAGE and immunoblotted with antibodies against total TrkA, TrkB, TrkC, phospholipase-Cγ (PLCγ), Protein kinase C (PKC)δ, PKCε, CREB, AKT and MAPK. All but TrkC were expressed in SY5Y cells.

Fig. 2: Time-course of Neurotrophin Signaling Activation following NGF treatment in SY5Y cells. Cells were incubated with 100 ng/ml NGF for the indicated time. Whole-cell extracts (30 mg total protein per lane) were resolved by SDS-PAGE and immunoblotted with antibodies against: A) phosphorylated (p-TrkA) and total TrkA; B) phosphorylated (p-MAPK) and total MAPK; or C) phosphorylated (p-PKC-δ) and total PKC-δ. Total TrkA, MAPK and PKC-δ were assessed using an antibody that recognizes TrkA, MAPK and PKC-δ regardless of the phosphorylation state.

Fig. 3: Concentration-dependent effects of thimerosal on neurotrophin signaling activation following NGF treatment in SY5Y cells. Cells were incubated with 100 ng/ml NGF and increasing concentrations of thimerosal for 5 minutes. Whole-cell extracts (30 mg total protein per lane) were resolved by SDS-PAGE and immunoblotted with antibodies against: A) phosphorylated TrkA (p-TrkA); B) phosphorylated MAPK (p-MAPK); or C) phosphorylated PKC-δ (p-PKC-δ). Results to the right of the blots are expressed as mean ± SD, n = 3-4. Blots on control gels were 1820 ± 590, 2380 ± 820, and 2080 ± 680 (optical density / sq. inch), mean ± SD, n = 3-4, respectively. For p-TrkA and p-MAPK,
concentrations >50 nM and 100 nM, respectively, were significantly different from control (p<0.05).

**Fig. 4: Thimerosal effects on cell viability.** SY5Y cells were exposed to increasing concentration of thimerosal in the presence (open symbols) and absence (filled symbols) of 100 ng/ml NGF for 24 (squares) or 48 hr (triangles). EC$_{50}$ values for cytotoxicity determined from the concentration-response curves for individual experiments using non-linear regression analysis were 596 and 38.7 nM in the presence and absence of NGF at 24 hr, respectively, and 105 and 4.35 nM in the presence and absence of NGF, respectively, at 48 hr. All EC$_{50}$ values were significantly different from one another.

**Fig. 5 Thimerosal alter DNA fragmentation in SY5Y cells.** SY5Y cells were exposed to increasing concentrations of thimerosal for 24 hrs in the absence (open diamonds) or presence (closed diamonds) of 100 ng/ml NGF. DNA fragmentation was measured by ELISA. The results are expressed as mean ± SE and representative of 2-3 independent measures. With or without NGF, values at 1 µM were significantly different from values at 0.01 µM.
Figure 2
Figure 3
Figure 5

Thimerosal Cytotoxicity: DNA Fragmentation

% Control

[Thimerosal] (uM)

0 0.001 0.01 0.1 1 10