

## Effects of Thimerosal on NGF Signal Transduction and Cell Death in Neuroblastoma Cells

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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  $\mu$ M), we measured the activation of TrkA, MAPK, and PKC- $\delta$ . In controls, the activation of TrkA MAPK and PKC- $\delta$  peaked after 5 min of exposure to NGF and then decreased but was still detectable at 60 min. Concurrent exposure to increasing concentrations of thimerosal and NGF for 5 min 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-h exposure to increasing concentrations of thimerosal, the EC<sub>50</sub> for cell death in the presence or absence of NGF was 596 nM and 38.7 nM, respectively. Following 48-h exposure to increasing concentrations of thimerosal, the EC<sub>50</sub> 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  $\mu$ M (apoptosis) to decrease at concentrations >1  $\mu$ M (necrosis). These data demonstrate that thimerosal could alter NGF-induced signaling in neurotrophin-treated cells at concentrations lower than those responsible for cell death.

**Key Words:** signal transduction; neurotrophin; mercury compound.

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 and Toyama, 1973; Ulfvarson, 1962), while the organic radical has a strong impact on both (Magos, 2003). Ethylmercury and its decomposition product, Hg<sup>2+</sup>, 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 h after incubation of human cortical neurons and fibroblasts with 250  $\mu$ M thimerosal. A 6 h 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.*, 2004a, 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

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