Thimerosal Induces Apoptosis in a Neuroblastoma Model via the cJun N-Terminal Kinase Pathway

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The cJun N-terminal kinase (JNK)-signaling pathway is activated in response to a variety of stimuli, including environmental insults, and has been implicated in neuronal apoptosis. In this study, we investigated the role that the JNK pathway plays in neurotoxicity caused by thimerosal, an ethylmercury-containing preservative. SK-N-SH cells treated with thimerosal (0–10µM) showed an increase in the phosphorylated (active) form of JNK and cJun with 5 and 10µM thimerosal treatment at 2 and 4 h. To examine activator protein-1 (AP-1) transcription, cells were transfected with a pGL2 vector containing four AP-1 consensus sequences and then treated with thimerosal (0–2.5µM) for 24 h. Luciferase studies showed an increase in AP-1 transcriptional activity upon thimerosal administration. To determine the components of the AP-1 complex, cells were transfected with a dominant negative to either cFos (A-Fos) or cJun (TAM67). Reporter analysis showed that TAM67, but not A-Fos, decreased AP-1 transcriptional activity, indicating a role for cJun in this pathway. To assess which components are essential to apoptosis, cells were treated with a cell-permeable JNK inhibitor II (SP600125) or transfected with TAM67, and the downstream effectors of apoptosis were analyzed. Cells pretreated with SP600125 showed decreases in activation of caspases 9 and 3, decreases in degradation of poly(ADP-ribose) polymerase (PARP), and decreased levels of proapoptotic Bim, in comparison to cells treated with thimerosal alone. However, cells transfected with TAM67 showed no changes in those same components. Taken together, these results indicate that thimerosal-induced neurotoxicity occurs through the JNK-signaling pathway, independent of cJun activation, leading ultimately to apoptotic cell death.

Key Words: thimerosal; mercury; neurotoxicity; JNK; cJun; Bim.

Thimerosal is an organic mercurial containing an ethylmercury moiety attached to the sulfur atom of thiosalicylate. Since the 1930’s, thimerosal has been used in many products as an antiseptic and a preservative. In recent years, controversy has surrounded the use of thimerosal in vaccines as mercury is a known neurotoxin and nephrotoxin. Since the controversy began in the late 1990’s, much of the thimerosal has been removed from vaccines administered to children in the United States. However, it remains in some, such as the influenza vaccine, and is added to multidose vials used in countries around the world. Studies concentrating on thimerosal-induced neurotoxicity are limited, and exposure guidelines, such as those set by the Food and Drug Administration, are based on research with methylmercury. Interestingly, some in vitro and in vivo studies suggest that ethylmercury may react differently than methylmercury (Aschner and Aschner, 1990; Harry et al., 2004; Magos et al., 1985). Few studies with thimerosal have focused on determining specific signaling pathways involved in neurotoxicity. Establishing these pathways may be an important step in discovering methods of alleviating toxic outcomes in patients exposed to thimerosal.

While the toxicological profile of thimerosal is still somewhat limited, the amount of information regarding thimerosal-induced toxicity is increasing. Recent studies have shown various events occurring in response to thimerosal exposure. Rat cerebellar neurons treated with thimerosal showed increases in intracellular calcium levels and decreases in glutathione levels (Ueha-Ishibashi et al., 2004a, 2005). Decreased glutathione levels resulting from thimerosal exposure were also seen in rat thymocytes (Ueha-Ishibashi et al., 2004b), cultured neuroblastoma cells (SH-SY5Y), and glioblastoma cells (James et al., 2005). HeLa S cells treated with thimerosal showed cytосkeletal changes and activation of focal adhesion kinase, both of which were attributed to the production of reactive oxygen species (Kim et al., 2002). Studies in our laboratory have begun to establish a more coordinated picture of events that occur in cells treated with thimerosal. We have shown that upon treatment with thimerosal, SK-N-SH neuroblastoma cells exhibited a time- and concentration-dependent increase in apoptotic cell death, as evidenced by increases in nuclear condensation, cytochrome c release, caspases 9 and 3 activation, poly(ADP-ribose) polymerase (PARP) degradation, and lactate dehydrogenase release, thus demonstrating a
mitochondrial-specific cell death pathway (Humphrey et al., 2005). The current study looks at upstream components in this apoptotic pathway.

One of the most likely candidates for apoptotic signaling pathways induced by thimerosal is the cJun N-terminal kinase (JNK)-activator protein-1 (AP-1) pathway. JNK phosphorylates AP-1 transcription factors, which can then proceed to bind DNA and alter transcription. The JNK-AP-1 pathway can be activated by cellular stresses and has been implicated specifically in neuronal apoptosis (Becker et al., 2004; Davis, 2000; Yang et al., 1997). Additionally, JNK and AP-1 have been linked to neuronal apoptosis through BiM, a proapoptotic, Bcl2-homology 3 (BH3)—only member of the Bcl2 family of proteins. Not only is BiM an AP-1 target gene but also it may be directly activated by JNK, leading to mitochondrial-mediated apoptosis (Becker et al., 2004; Harris and Johnson, 2001; Lei and Davis, 2003).

In this study, we use the SK-N-SH human neuroblastoma cell line to investigate signaling events occurring during thimerosal-induced neurotoxicity. Here we report that JNK and cJun are activated in SK-N-SH cells following thimerosal treatment. By using a cell-permeable JNK inhibitor (SP600125) to block the pathway, we demonstrate that JNK activation is an essential component of thimerosal-induced cell death. However, through the use of a cJun dominant negative, we show that cJun activation is not required for cell death in SK-N-SH cells treated with thimerosal. To our knowledge, this is the first study to show that thimerosal activates JNK, leading to cell death in a cJun-independent manner. These results are significant because they establish a signaling pathway that could be targeted pharmacologically to decrease toxicity in patients exposed to thimerosal.

**MATERIALS AND METHODS**

**Chemicals.** Thimerosal (minimum 97% HPLC), thiosalicylate (minimum 95%), Ponceau S, 12-O-tetradecanoylphorbol 13-acetate (TPA), and sodium pyruvate were purchased from Sigma-Aldrich Chemical Corp. (St Louis, MO).

**Cell culture and treatment.** SK-N-SH neuroblastoma cell line was purchased from American Type Culture Collection and maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, 1% antibiotics (penicillin/streptomycin/neomycin, Invitrogen Corp., Carlsbad, CA), and 1mM sodium pyruvate. Cells were incubated at 37°C in a humidified atmosphere of 5% CO2. Cells were treated with a range of thimerosal concentrations, and thiosalicylate was used as a control to ensure that changes noted following treatment were attributed to the mercury-containing portion of the thimerosal molecule. Additional experiments were also carried out using 10μM JNK inhibitor II (SP600125, Calbiochem, La Jolla, CA) prior to thimerosal treatment. SP600125 is an anthrapyrazolone compound that is a JNK-specific, reversible, ATP-competitive inhibitor (Bennett et al., 2001). Preliminary studies were conducted to determine the lowest concentration of SP600125 needed to block thimerosal-mediated phosphorylation of cJun.

**Western analysis.** Cytoplasmic samples (p-JNK, c-Jun, cFos, p-cJun [Ser63], p-cJun [Ser73], and β-actin), or whole-cell lysates (Bim/BOD, cleaved caspase 3, caspase 9, PARP, and GAPDH) were run on a 12.5% SDS-PAGE according to the method of Laemmli (1970) and transferred to nitrocellulose membrane. Transfer efficiency was assessed by staining with 0.1% Ponceau S, and then blots were washed with distilled water to remove stain. Blots were blocked for 1 h at room temperature in Bboto (5% milk, 10mM Tris-HCl, 150mM NaCl [pH 8.0], 0.05% Tween-20) or 5% milk solution in TBST (10mM Tris-HCl, 150mM NaCl, 0.1% Tween-20 [pH 8.0]; p-JNK, p-cJun [Ser63], p-cJun [Ser73]). Each blot was incubated overnight at 4°C with the primary antibody at a dilution of 1:1000 in Bboto (cJun, cFos, Santa Cruz Biotechnology, Santa Cruz, CA; Bim/BOD, Stressgen, Victoria, Canada; cleaved caspase 3, caspase 9, and PARP, Cell Signaling Technology, Inc., Beverly, MA; GAPDH [1:10,000], Trevigen, Gaithersburg, MD; β-actin [1:10,000], Sigma-Aldrich Chemical Corp.) or 5% bovine serum albumin in TBST (p-JNK, p-cJun [Ser63], p-cJun [Ser73], Cell Signaling Technology, Inc.). The next day, each blot was incubated with an anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) at a 1:3000 dilution or anti-mouse IgG-HRP (Santa Cruz Biotechnology) at a 1:10,000 dilution (β-actin) in Bboto or 5% milk solution in TBST (p-JNK, p-cJun [Ser63], p-cJun [Ser73]) for 1.5 h at room temperature. Protein bands were then visualized using the enhanced chemiluminescence detection system (Amersham, Little Chalfont, U.K.). All Western analysis experiments were repeated at least three times.

**Transient transfection.** Cells were plated at a density of 3 × 104 cells per 100-mm plate and grown overnight. Cells were transfected using the calcium phosphate method and were transfected with the pGL2 empty vector or with 1μM of the AP-1 construct or cotransfected with the AP-1 construct and either A-Fos (0.125 or 0.25 μg) or TAM67 (0.25 or 0.5 μg). The AP-1 construct consists of four consensus sequences inserted into the pGL2 vector (Promega, Madison, WI). The A-Fos construct is a dominant negative consisting of an N-terminal acidic extension added to cFos and cloned into the CMV-500 vector (Steinmüller et al., 2001). This construct was a generous gift from Dr Charles Vinson (National Institutes of Health). The TAM67 construct is a cJun dominant negative that lacks the transactivation domain and was cloned into the pcDNA3.1(–) vector (Brown et al., 1994). This construct was a generous gift from Dr Michael Birrer (National Institutes of Health). Both dominant negative forms retain the ability to dimerize with cJun and cFos proteins and bind DNA; however, structural alterations prevent AP-1–mediated transcriptional events. Seven hours after transfection, cells were washed twice with phosphate-buffered saline (PBS; 137mM sodium chloride, 3mM potassium chloride, 1mM potassium phosphate, and 10mM sodium phosphate), and fresh medium was added. On the following day, cells were trypsinized and plated in 24-well plates at a density of 1.5 × 104 cells per well. Twenty-four hours later, cells were treated in triplicate with 0–2.5μM thimerosal or 100nM TPA. After 24 h, cells were washed with PBS and lysed using 200 μl of passive lysis buffer (Promega). Reporter assays were performed using the luciferase assay system (Promega) and TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

Additional experiments were conducted using cells cotransfected with AP-1 and 0.5 μg TAM67. Following transfection as above, these cells were plated in 100-mm dishes at a density of 1 × 105 cells per plate. After 24 h, cells were treated with 2.5μM of either thiosalicylate (control) or thimerosal for 24 h, and then whole-cell lysates were collected and analyzed by Western blotting. All transient transfection experiments were performed at least three times.

**Statistical analysis.** Statistical analysis was performed using SigmaStat software (SPSS, Inc. Chicago, IL). Results were assessed with analysis of variance and Dunnett’s test with a p value of 0.05.

**RESULTS**

**Thimerosal Induces Phosphorylation of JNK**

The JNK pathway is activated by cellular stresses, such as thimerosal, resulting in the phosphorylation of JNK, which
then phosphorylates proteins, including transcription factors. SK-N-SH cells, treated with a range of thimerosal concentrations (0–10 μM) for 2 and 4 h, showed an increase of JNK phosphorylation at 5 and 10 μM at both time points in comparison with thiosalicylate (Fig. 1). The activated JNK was present in both the cytoplasm (Fig. 1A) and the nucleus (Fig. 1B). Multiple bands are present because the three JNK genes encode 10 JNK isoforms, and these splice variants migrate at either 46 or 54 kDa (Davis, 2000). Lower concentrations of thimerosal required longer incubation times to activate JNK. For instance, cells treated with 2.5 μM thimerosal showed increases in p-JNK that were increased over control at 6 h (Fig. 1C). This mitogen-activated protein kinase induction was specific for JNK; p38 and extra cellular signal-regulated protein kinase (ERK) were not activated in response to thimerosal exposure. Additionally, total JNK levels remained unchanged (data not shown). Activated JNK in the nucleus is significant because this is a potential site of interaction with transcription factors, including AP-1. Activated JNK in the cytoplasm is also important because this is a second site of interaction with cellular components, including proteins mediating apoptosis.

**Thimerosal Treatment Causes Increases in cJun and cFos**

AP-1 most often consists of dimerized proteins from the cJun and cFos families. Transcription factors from the cJun family can form either homodimers or heterodimers with members of the cFos family. To determine if protein levels of either cJun or cFos were increased in response to thimerosal exposure, we treated SK-N-SH cells with a range of thimerosal concentrations (0–10 μM) for 2 and 4 h and collected nuclear extracts. Western blotting of nuclear extracts showed that cells treated with 10 μM for 2 h had increases in total cJun and cFos levels (Fig. 2). Extending treatment time to 4 h showed increases in cJun levels with 2.5–10 μM thimerosal treatment in comparison to control (Fig. 2A), while cFos levels were increased with 5–10 μM thimerosal treatment (Fig. 2B). However, increased protein levels do not necessarily indicate activation of transcription factors, which must first be phosphorylated to be activated.

**Thimerosal Induces Phosphorylation of cJun (Ser63 and Ser73)**

The activation of cJun was determined by assessing the immunoreactivity of phosphorylated cJun following thimerosal treatment. Nuclear extracts of SK-N-SH cells treated with a range of thimerosal concentrations (0–10 μM) for 2 and 4 h showed an increase in cJun phosphorylation at 5 and 10 μM at both time points in comparison with the control (Fig. 1). The activated JNK was present in both the cytoplasm (Fig. 1A) and the nucleus (Fig. 1B). Multiple bands are present because the three JNK genes encode 10 JNK isoforms, and these splice variants migrate at either 46 or 54 kDa (Davis, 2000). Lower concentrations of thimerosal required longer incubation times to activate JNK. For instance, cells treated with 2.5 μM thimerosal showed increases in p-JNK that were increased over control at 6 h (Fig. 1C). This mitogen-activated protein kinase induction was specific for JNK; p38 and extra cellular signal-regulated protein kinase (ERK) were not activated in response to thimerosal (data not shown). Activated JNK in the nucleus is significant because this is a potential site of interaction with transcription factors, including AP-1. Activated JNK in the cytoplasm is also important because this is a second site of interaction with cellular components, including proteins mediating apoptosis.

**Thimerosal Activates AP-1 Transcriptional Activity through cJun but not cFos**

To further investigate the activation of the JNK-AP-1 pathway, we next looked at AP-1 transcriptional activity. We transfected SK-N-SH cells with the pGL2 empty vector or the AP-1 construct using a luciferase reporter assay system. Cells treated with 2.5 μM thimerosal showed a significant increase in
luciferase activity, indicating an increase in AP-1 transcriptional activation. Transfected cells were also treated with 100nM TPA, a known activator of AP-1 transcriptional activity that served as a positive control (Figs. 4 and 5). We next used the A-Fos dominant negative to determine the role of cFos in this pathway. Cells transfected with the AP-1 construct alone or cotransfected with (0.125 or 0.25 μg) A-Fos showed comparable increases in luciferase activity, suggesting that cFos is not part of this AP-1 complex activated by thimerosal (Fig. 4). The TPA-induced increase in luciferase activity was inhibited, indicating that the A-Fos construct was able to block AP-1 transcriptional activity (Fig. 4). The TAM67 dominant negative was used to determine the role of cJun in this pathway. Cells cotransfected with the cJun dominant negative and AP-1 construct did not show increases in luciferase activity following thimerosal treatment, indicating blockage of AP-1 transcriptional activity (Fig. 5). This suggests that cJun is playing an active role in thimerosal-induced AP-1 transcriptional activation, possibly by forming a homodimer.

### Increases in Proapoptotic Bim Levels after Thimerosal Treatment

Bim is a proapoptotic member of a subgroup of the Bcl2 family of proteins that contain only the BH3 domain. These proteins interact with and inactivate antiapoptotic Bcl2 family members in response to cellular stress, such as DNA damage (Bouillet et al., 2001). Bim may also be directly activated by JNK, leading to mitochondrial-mediated apoptosis. Additionally, Bim is an AP-1 target gene; therefore, we wanted to determine if Bim was associated with thimerosal-induced toxicity in this cell line. In cells treated with 2.5μM thimerosal for 24 h, Western blotting showed that Bim levels were increased (Fig. 6A, lane 3) in comparison with control (Fig. 6A, lane 1).

### Pretreatment with JNK Inhibitor II (SP600125) Blocks Increases in Bim and Other Apoptotic Proteins

To confirm that JNK is an essential component of this thimerosal-induced, mitochondrial-mediated apoptotic pathway, we chose to use the cell-permeable JNK inhibitor II (SP600125). By blocking the activation of JNK and its subsequent ability to activate transcription factors and other cellular proteins, we can determine if it is playing a role in thimerosal-mediated apoptosis. SK-N-SH cells were pretreated
for 2 h with 10μM SP600125 followed by 2.5μM thimerosal for 24 h, as above, and then examined with Western blotting. Cells pretreated with the inhibitor followed by thimerosal administration showed less of an increase in levels of Bim (Fig. 6A), cleaved caspase 9 (Fig. 6B), cleaved caspase 3 (Fig. 6C), and PARP (Fig. 6D) than did cells treated with thimerosal alone, indicating that blockage of the JNK pathway has a protective effect on the cells. To confirm this, cells were examined microscopically for morphological changes. The SP600125-pretreated cells showed morphology similar to control cells, while those treated with thimerosal alone showed morphological changes evident of toxicity, as previously described (Humphrey et al., 2005; Fig. 7).

**FIG. 6.** (A–D) Lane 1 represents SK-N-SH cells pretreated for 2 h with vehicle (100% DMSO) followed by 24 h treatment with 2.5μM thiosalicylate. Lane 2 represents cells pretreated for 2 h with 10μM SP600125 followed by 24 h treatment with 2.5μM thiosalicylate. Lane 3 represents cells pretreated for 2 h with vehicle (100% DMSO) followed by 24 h treatment with 2.5μM thimerosal. Lane 4 represents cells pretreated with 10μM SP600125 followed by 24 h treatment with 2.5μM thimerosal. Western blotting was performed on whole-cell lysates to assess changes in levels of Bim (A), caspase 9 (B), caspase 3 (C), and PARP (D). A total of 50 μg of whole-cell lysates was loaded per lane. GAPDH was used as a loading control.

**Blockage of cJun Transcriptional Activity Does Not Prevent Apoptosis Following Thimerosal Treatment**

After we established that JNK was essential to thimerosal-induced neuronal apoptosis, we looked to see if this response was also dependent on cJun activation. Cells were again transfected with TAM67, and whole-cell lysates were analyzed by Western blotting for changes in apoptotic parameters.

**FIG. 7.** Cells were pretreated for 2 h without (A and C) or with (B and D) SP600125 and treated with 2.5μM thiosalicylate (A and B) or 2.5μM thimerosal (C and D) and then observed microscopically for morphological changes. Cells were observed with a Nikon Diaphot (Frank E. Fryer Co., Cincinnati, OH) and photographed using a Nikon N2000 camera.
showed that primary cultures of rat cerebellar neurons undergo apoptotic cell death in cultured astrocytes. Kunimoto (1994) demonstrated that mercuric chloride causes disruption of these components can lead to cell death. Monnet-Tschudi (1998) showed that primary cultures of rat cerebellar neurons undergo apoptosis when exposed to low concentrations of methylmercury but undergo necrotic cell death when exposed to higher concentrations. Additionally, several studies have shown that once organic mercurials enter the body, they can be converted to inorganic mercury, which has a much longer half-life than the parent compound (Friberg and Mottet, 1989; Norseth and Clarkson, 1970; Suda et al., 1991). Several studies have indicated that both inorganic and organic mercurials induce apoptosis in vitro as well as in vivo (Guo et al., 1998; Shenk et al., 1999, 2000). At the organ level, one of the primary targets for mercurials is the brain. Studies using Wistar rats exposed to methylmercury demonstrated that cerebellar granule cells underwent apoptotic cell death (Nagashima et al., 1996). Baskin et al. (2003) demonstrated that thimerosal-induced changes in membrane permeability, DNA damage, and caspase 3 activation in both cultured human cerebral cortical neurons and fibroblasts. It has been known for 30 years that mercury accumulates in rabbit brain tissue following the application of thimerosal-containing ophthalmic medications (Gassett et al., 1975) and that inorganic mercury accumulates in monkey brain tissue following daily administration of thimerosal (Blair et al., 1975). Recent studies have shown that mercury accumulates in the brain tissue of neonatal mice given intramuscular injections of thimerosal, and the levels did not decrease at 7 days after treatment (Harry et al., 2004). Hornig et al. (2004) used an autoimmune disease-sensitive mouse model (SJL/J) to show that some populations may have a genetic predisposition for sensitivity to thimerosal-induced neurotoxicity. However, specific signaling pathways in thimerosal-induced neurotoxicity have not been established in animal or tissue culture models.

Untransfected cells and those transfected with empty vectors or AP-1 construct showed increases in caspase 3 cleavage when treated with thimerosal in comparison to thiosalicylate (Fig. 8). Cells transfected with TAM67 still underwent apoptosis when treated with 2.5 μM thimerosal, as seen by similar increases in cleaved caspase 3 (Fig. 8), cleaved caspase 9, Bim, and PARP (data not shown). These results indicate that cJun activation is not an essential component of apoptosis in response to thimerosal exposure.

**DISCUSSION**

The present study demonstrates for the first time the essential role of JNK activation in thimerosal-induced cell death in an in vitro model. The SK-N-SH cell line was chosen due to cellular characteristics representative of those in an immature nervous system. The SK-N-SH cell line, comprised a neuroblast cell type, retains the capacity to differentiate in vitro, rather than representing a model of terminal differentiation. In addition, neuroblastoma models have been reported to be sensitive to mercurial toxicity (Sager and Syversen, 1984; Stoiber et al., 2004; Toimela and Tahti, 2004). Limited studies have been conducted to test actual mercury levels in infants immediately following administration of thimerosal-containing vaccines. Consequently, in our initial studies, such as those looking at JNK and cJun activations, we used a range of concentrations (0–10 μM) for short time periods to conduct these mechanistic studies in our model. Because the higher concentrations of thimerosal were so toxic and cells died too quickly to measure luciferase activity, we used lower concentrations (0–2.5 μM) for longer time periods in order to establish molecular events occurring following thimerosal treatment.

At the cellular level, mercurials readily react with sulfhydryl groups, making enzymes and structural proteins likely targets. Disruption of these components can lead to cell death. Monnet-Tschudi (1998) demonstrated that mercuric chloride causes apoptotic cell death in cultured astrocytes. Kunimoto (1994) showed that primary cultures of rat cerebellar neurons undergo apoptosis when exposed to low concentrations of methylmercury but undergo necrotic cell death when exposed to higher concentrations. Additionally, several studies have shown that once organic mercurials enter the body, they can be converted to inorganic mercury, which has a much longer half-life than the parent compound (Friberg and Mottet, 1989; Norseth and Clarkson, 1970; Suda et al., 1991). Several studies have indicated that both inorganic and organic mercurials induce apoptosis in vitro as well as in vivo (Guo et al., 1998; Shenk et al., 1999, 2000). At the organ level, one of the primary targets for mercurials is the brain. Studies using Wistar rats exposed to methylmercury demonstrated that cerebellar granule cells underwent apoptotic cell death (Nagashima et al., 1996). Baskin et al. (2003) demonstrated that thimerosal-induced changes in membrane permeability, DNA damage, and caspase 3 activation in both cultured human cerebral cortical neurons and fibroblasts. It has been known for 30 years that mercury accumulates in rabbit brain tissue following the application of thimerosal-containing ophthalmic medications (Gassett et al., 1975) and that inorganic mercury accumulates in monkey brain tissue following daily administration of thimerosal (Blair et al., 1975). Recent studies have shown that mercury accumulates in the brain tissue of neonatal mice given intramuscular injections of thimerosal, and the levels did not decrease at 7 days after treatment (Harry et al., 2004). Hornig et al. (2004) used an autoimmune disease-sensitive mouse model (SJL/J) to show that some populations may have a genetic predisposition for sensitivity to thimerosal-induced neurotoxicity. However, specific signaling pathways in thimerosal-induced neurotoxicity have not been established in animal or tissue culture models.

Previous studies in our laboratory indicated that thimerosal-induced cell death occurs via a mitochondrial mechanism (Humphrey et al., 2005). However, the upstream mediators were not known. In this study, we find that the JNK pathway is activated following thimerosal treatment, as seen by increases in phosphorylation of JNK. We also see an increase in cJun activation, followed by increases in AP-1 transcriptional activity, indicating a possible role for AP-1 in thimerosal-induced cell death. However, studies with the TAM67 dominant negative show that when cJun is unable to activate transcription, SK-N-SH cells treated with thimerosal still undergo apoptosis, as shown by the activation of caspases 9 and 3 and degradation of PARP. These results suggest that cJun activation is not required for thimerosal-induced cell death. Therefore, the AP-1 transcription factor must have another role following this environmental insult. Following cellular damage, cells enter into a period of cell cycle arrest in order to repair damage. Shaulian et al. (2000) found that induction of cJun is required for reentry into the cell cycle following UV damage. In the case of thimerosal exposure, we saw increases in levels of cJun, which can be increased in response to AP-1 activation, occurring at a later time point than JNK and cJun activations. This protein increase could be the result of an
attempt by the cell to prepare to reenter the cell cycle following repair. cJun can stimulate reentry into the cell cycle through induction of cyclin D1, which drives cell proliferation, or through repression of p21Cip1, which promotes p53 activity (Shaulian et al., 2000). On the other hand, Shaulian and Karin (2002) suggest that the role of cJun in apoptosis and survival might be more of a balancing act between cJun-mediated induction of proapoptotic and antiapoptotic target genes, including FasL, Bcl3, and Bim. Depending on cell type, the nature of the stress, duration of exposure, and other active transcription factors, the balance may shift toward either survival or apoptosis. Further studies will be needed to determine if either of these events is occurring in response to thimerosal since SK-N-SH cells treated with the mercury-containing compound appear unable to successfully process the toxic insult and thus proceed down an apoptotic pathway.

Our studies are the first to show that proapoptotic Bim levels are increased in response to thimerosal treatment and are decreased when cells were pretreated with SP600125 but not when transfected with TAM67, indicating that perhaps JNK and Bim are interacting directly. JNK has been shown to activate Bim through phosphorylation, allowing Bim to contribute to apoptosis by interaction with other Bcl2 proteins (Becker et al., 2004; Harris and Johnson, 2001). Under normal conditions, Bim is attached to LC8 in the dynein motor complex but translocates to the mitochondrial membrane when activated, wherein Bim interacts with Bax/Bad, causing pore formation and cytochrome c release, ultimately leading to apoptosis (Mollinedo and Gajate, 2003). According to Putcha et al. (2001), this Bim-mediated apoptosis is specific to neuronal cells and does not occur in nonneuronal cells in the brain. Previous studies in our laboratory have shown that thimerosal-treated SK-N-SH neuroblastoma cells have increases in cytochrome c release, so this interaction with Bim is a potential mechanism by which JNK could be directly causing apoptosis in these cells. Additionally, Bim is an AP-1 target gene, but after blocking AP-1 transcription, we still see increases in levels of Bim, indicating that in SK-N-SH cells Bim levels may be increased in response to other transcription factors, such as members of the Forkhead family (Gilley et al., 2003; Sunters et al., 2003). Essers et al. (2004) found that JNK can phosphorylate Forkhead transcription factors, leading to apoptotic cell death. Further studies need to be conducted to determine the significance of both the AP-1 activation and the substrates through which JNK is mediating intrinsic apoptotic cell death, including the investigation of the JNK-Bim interaction.

Our study is the first demonstration that thimerosal can induce the activation of JNK and AP-1 in the SK-N-SH neuroblastoma cell line. We showed that activation of cJun and AP-1 transcriptional activity following thimerosal treatment does not appear to be involved in the induction of apoptosis, as demonstrated with the studies using the cJun dominant negative. Furthermore, we were able to show that JNK is an essential upstream component of this pathway through the use of the JNK inhibitor SP600125. This compound was able to attenuate activation of downstream components of mitochondrial-mediated cell death and subsequently protect the cells from apoptosis. These results are significant because identifying specific signaling pathways activated in response to thimerosal exposure presents pharmacological targets for attenuating potential toxicity in patients exposed to thimerosal-containing products.

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