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Dibutyltin Activates MAP kinases in Human Natural Killer Cells, *in vitro*

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Abstract

Previous studies have shown that dibutyltin (DBT) interferes with the function of human natural killer (NK) cells, diminishing their capacity to destroy tumor cells, *in vitro*. DBT is a widespread environmental contaminant and has been found in human blood. As NK cells are our primary immune defense against tumor cells, it is important to understand the mechanism by which DBT interferes with their function. The current study examines the effects of DBT exposures on key enzymes in the signaling pathway that regulates NK responsiveness to tumor cells. These include several protein tyrosine kinases (PTKs), mitogen activated protein kinases (MAPKs) and mitogen activated protein kinase kinases (MAP2Ks). The results showed that *in vitro* exposures of NK cells to DBT had no effect on PTKs. However, exposures to DBT for as little as 10 min were able to increase the phosphorylation (activation) of the MAPKs. The DBT-induced activations of these MAPKs, appears to be due to DBT-induced activations of the immediate upstream activators of the MAPKs, MAP2Ks. The results suggest that DBT-interference with the MAPK signaling pathway is a consequence of DBT exposures, which could account for DBT-induced decreases in NK function.

Keywords

MAP kinases; MAP kinase kinases; Protein tyrosine kinases; NK cells; Dibutyltin

INTRODUCTION

Dibutyltin (DBT) has been used as a stabilizer in polyvinyl chloride (PVC) plastic products (Roper, 1992) and as a de-worming agent in poultry (Epstein et al., 1991). Due to these uses it has been found in some plastic food containers (Nakashima et al., 1990; Yamada et al., 1993) as well as some poultry products (Epstein et al., 1991). DBT has also been found in drinking water due to leaching from PVC pipes (Sadiki et al., 1996). Human exposure to DBT could result from poultry products (Epstein et al., 1991), consuming beverages stored in PVC pipes during manufacture (Forsyth et al., 1992a,b), and drinking water (Sadiki et al., 1996). DBT has been found in human blood from donors with no known route of exposure other than what is found in the environment (Kannan et al., 1999; Whalen et al., 1999). A Danish study found DBT in liver samples from eighteen deceased Danish males who had no documented exposure to DBT (Nielsen and Strand, 2002).

DBT appears to cause a variety of birth defects in rats and to disrupt normal embryonic development (Ema et al., 1996; Noda et al., 1993). Thymus atrophy and pancreatitis are

consequences of DBT exposure in rats (Merkord et al., 1997; Pieters et al., 1994). Exposure of animals to DBT may debilitate their immune function, rendering them more vulnerable to infectious diseases (Kannan et al. 1997, 1998).

The innate immune system includes natural killer (NK) lymphocytes, which have a critical role in protecting the body against viral infections and tumor growth (Trinchieri, 1989; Biron, 1997). Therefore, suppressing their cytotoxic function could debilitate the immune system's ability to eradicate viral infections and tumors. In previous studies we have shown that exposure of human NK cells to DBT, *in vitro*, causes a very significant suppression of their ability to kill tumor cells (Dudimah et al., 2007; Whalen et al., 1999; Whalen et al., 2002).

When an NK cell binds to a target cell, non-receptor protein tyrosine kinases (PTKs) are activated (Gismondi *et al.*, 2000). Activation of PTKs causes activation of guanine nucleotide exchange factors (GEFs), and subsequent activation of small GTP-binding (G) proteins (Vivier *et al.*, 2004; Djeu *et al.*, 2002). Activation of small G-proteins lead to activation of mitogen-activated protein kinases (MAPKs), which include p44/42 (also known as ERK1/2), p38, and JNK. Activation of p44/42 appears to be a part of the mechanism which controls the release of cytotoxic granules (Trotta *et al.*, 1998, Trotta *et al.*, 2000, Wei *et al.*, 1998). p38 and JNK appear to regulate the responsiveness of the NK cell (Trotta *et al.*, 2000; Chan et al., 2001). MAPKs are activated by phosphorylation catalyzed by mitogen-activated protein kinase kinases (MAP2Ks). p44/42 is activated by an upstream MAP2K, MEK1/2 (Derijard *et al.*, 1995; Payne *et al.*, 1991; Han *et al.*, 1996) while p38 is activated by MKK3/6 (Han *et al.*, 1996). MAP2Ks are activated by mitogen-activated protein kinase kinase kinases (MAP3Ks) (Vivier *et al.*, 2004).

As DBT has been shown to suppress human NK function, it is important to delineate the mechanism by which this suppression occurs. Activation of any of the enzymes of the above described pathway, such as MAPKs, by DBT would leave the NK cell unable to respond to subsequent target cell contact, as the enzyme(s) would not be available for activation by target cells. It is known that prior activation of the enzymes of the cytotoxic pathway leaves the NK cell unable to respond to subsequent target cell encounters (anergic) (Jewett and Bonavida, 1995).

In the previous studies we have shown that exposure of NK cells to another organotin compound, tributyltin (TBT), caused activation of the MAPKs, p44/42, p38, and JNK (Aluoch and Whalen, 2005; Aluoch et al., 2006; Aluoch et al., 2007). Here we investigate the effects of exposures to DBT on the activation states of PTKs, MAP2Ks, and MAPKs in human NK cells.

MATERIALS AND METHODS

Isolation of NK cells

Peripheral blood from healthy adult (male and female) volunteer donors was used for this study (American Red Cross, Portland OR, Key Biologics, Memphis, TN). Highly enriched NK cells were obtained using a rosetting procedure. The buffy coats were fresh having been collected within 24 h of the NK-cell isolation procedure. Buffy coats were mixed with 0.6–1 mL of RosetteSep human NK cell enrichment antibody cocktail (StemCell Technologies, Vancouver, British Columbia, Canada) per 45 mL of buffy coat. The mixture was incubated for 30 min at room temperature (~ 25° C). Following the incubation, 6–8 mL of the mixture was layered onto 4 mL of Ficoll-Hypaque (1.077 g/mL) (Sigma) and centrifuged at 1200 g for 30–40 min. The cell layer was collected and washed twice with PBS (phosphate buffered saline, pH 7.2) and stored in complete media (RPMI-1640 supplemented with 10% heat-inactivated BCS (bovine calf serum), 2 mM L-glutamine and 50 U penicillin G with 50 µg streptomycin/ml) at

1 million cells/mL. The resulting cell preparation was >95% CD16+, 0% CD3+ by fluorescence microscopy (Whalen *et al.*, 2002).

Chemical Preparation

Dibutyltin was purchased from Aldrich Chemical Co. (Milwaukee, WI). A DBT stock solution was prepared by dissolving DBT in dimethylsulfoxide (DMSO). This DBT stock solution was diluted media to achieve final concentrations of 10-0.5 μ M.

Cell Lysates

NK cells were treated with 10-0.5 μ M DBT or control for 10 min, 60 min, or 6 h. Following the treatments, the cells were centrifuged and the cell pellets were lysed using 500 μ L of lysis buffer (Active motif, Carlsbad, CA) per 10 million cells. The cell lysates were stored frozen at -80°C up to the point when they were run on SDS-PAGE. All controls and DBT-exposed cells for a given experimental set-up (described above) were from an individual donor. Each of the experimental set-ups was repeated a minimum of three times using cells from different donors.

Western Blot

Cell lysates were run on 10% SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) and transferred to PVDF (polyvinylidene Difluoride) membrane. The PVDF was immunoblotted with specific primary antibodies listed in table 1. Antibodies were visualized using the ECL chemiluminescent detection system (Amersham, Piscataway, NJ) and a Kodak Image Station (Kodak, Rochester, NY). The density of each protein band was determined by densitometric analysis using the Kodak Image Station analysis software. The settings on the image station were optimized to detect the largest possible signal range and to prevent saturation of the system. A given experimental set-up (as described in the cell lysate section) always had its own internal control. Thus, differences in phosphorylation state and changes in protein expression were determined relative to the internal control. This determination provided relative quantitation by evaluating whether a given treatment altered these enzymes/proteins relative to untreated cells. β -actin levels were determined for each condition to verify that equal amounts of protein were loaded. In addition, the density of each protein band was normalized to β -actin to correct for small differences in protein loading among the lanes.

Phosphatase Assay

A serine/threonine phosphatase assay kit was purchased from AnaSpec, Inc. (San Jose, CA). Total ser/thr phosphatase activity in control and DBT-exposed NK cells was measured by the addition of 50 μ L pNPP reaction mixture containing (12.5 μ L of pNPP stock solution (100 $\times$), 1.24 mL of assay buffer and 3.75 μ L of 1M DTT) to the appropriate dilutions of the cell lysates (50 μ L) in the wells of a 96-well plate. The plate was incubated at room temperature and absorbance was measured at 405 nm in a microplate reader every 5 min for 40 min.

Statistical analysis

Analysis of variance (ANOVA) followed by pairwise comparison of data was carried out for all studies. A significant ANOVA was followed by pair wise comparisons of control versus DBT-exposed data using a *t*-test. A minimum of three separate determinations were carried out for each Western blot experimental set-up ($n \geq 3$) and statistical significance was noted at $p < 0.05$.

RESULTS

Effects of 10 min exposures to 10-0.5 μ M DBT on phosphorylation of p38 and MKK3/6 in NK cells

Figure 1 shows the effects of 10 min exposures to DBT on the phosphorylation of p38 and its immediate upstream activator MKK3/6. Significant increases in phospho-p38 of 1.8, 2.2, 1.8, 1.6 and 1.5-fold (compared to control) were seen at 10, 5, 2.5 and 0.5 μ M DBT, respectively ($p < 0.05$). The observed activation (phosphorylation) of p38 prompted us to investigate the effects of DBT on its upstream activator, MKK3/6. Exposure to 10, 5, 2.5, and 0.5 μ M DBT also produced significant increases in phospho-MKK3/6 of 1.8, 1.8, 1.60, and 1.8-fold compared to control, respectively ($p < 0.05$) (Figure 1). The increases in phosphorylation of MKK3/6 seen with 1 μ M DBT ranged from 1.3 to 2.5-fold with an average value of 1.6-fold, however due to the variability of the increase the p value was greater than 0.05. Thus, for every p38 activation, there was a corresponding activation of MKK3/6. Figure 1A shows the fold increases in phosphorylation of p38 and MKK3/6 combined from at least 3 separate experiments. Figures 1B and 1C show data from representative experiments. The total levels of p38 and MKK3/6 were not significantly altered by exposures to DBT (data not shown).

Effects of 10 min exposures to 10-0.5 μ M DBT on phosphorylation of p44/42 and MEK1/2

Figure 2 shows changes in phospho-p44/42 and phospho-MEK1/2 in NK cells exposed to DBT for 10 min. Significant increases in phospho-p44/42 of 1.8-fold at 10 μ M DBT and 1.6-fold at 5 μ M DBT compared to control were seen following 10 min exposures ($p < 0.05$). No significant activation was seen at 2.5-0.5 μ M DBT. There was a corresponding activation of the upstream activator of p44/42, MEK1/2. Exposure to 10 μ M DBT caused a 1.9-fold increase in phosphorylation of MEK1/2 while 5 μ M DBT caused a 2-fold increase ($P < 0.05$). Figure 2A shows the fold increases in phosphorylation of p38 and MKK3/6 combined from at least 3 separate experiments. Figures 2B and 2C show data from representative experiments. As with p38 and MKK3/6 there was no significant increase in the total levels of p44/42 or MEK1/2 with DBT exposures (data not shown).

Effects of 10 min exposures to 10-0.5 μ M DBT on the phosphorylation of JNK and MKK4

No significant changes in the phosphorylation of JNK or its MAP2K, MKK4 were observed following 10 min exposures of NK cells to 10-0.5 μ M DBT (Figure 3A and B).

Effects of 10 min exposures to 10-0.5 μ M DBT on non-receptor protein tyrosine kinases (PTKs)

No significant activations of any PTKs (Zap-70, Syk, Src or Pyk2) were observed within 10 min of exposure to 10-0.5 μ M DBT (Figure 3C).

Effects of 60 min exposures to 10-0.5 μ M DBT on p38, MKK3/6, p44/42, MEK1/2, JNK, and MKK4

Figure 4 shows the effects of 60 min exposures to DBT on the phosphorylation of p38 and its MAP2K, MKK3/6. Significant increases in phosphorylation of the MAPK, p38, of 2.3, 2.0, 1.8 and 1.5-fold compared to control, were seen at 10, 5, 2.5, and 1 μ M DBT, respectively ($p < 0.05$) (Figure 4A and B). As with 10 min exposures, there were corresponding activations of MKK3/6. Phosphorylation of MKK3/6 was increased 1.6, 1.6, 1.9 and 1.6-fold compared to control at 10, 5, 2.5, and 1 μ M DBT, respectively ($p < 0.05$) (Figure 4A and C). Again, there were no increases in the total levels of either p38 or MKK3/6 (data not shown). When NK cells were exposed to 10-0.5 μ M DBT for 1 h there were no significant alterations of the phosphorylation state of p44/42 or its MAP2K, MEK1/2 (Figure 4D and E). Likewise there were no significant changes in the total levels of p44/42 (data not shown). No significant

changes in the phosphorylation of the MAPK, JNK, or its MAP2K, MKK4, were observed following 60 min exposures of NK cells to 10-0.5 μ M DBT (data not shown).

Effects of 6 h exposures to 10-0.5 μ M DBT on p38, MKK3/6, p44/42, MEK1/2, JNK, and MKK4

Changes in the phosphorylation state of p38 and MKK3/6 in NK cells exposed to DBT for 6 h are shown in Figure 5A–C. There were significant increases in phospho-p38 (2.2-fold) compared to control ($p < 0.05$) at 10 μ M DBT. There were no significant increases in phosphorylation seen at any of the other concentrations. Exposure to 10 μ M DBT also significantly increased the phosphorylation of MKK3/6 (1.5-fold). Again there were no significant changes in the total levels of p38 or MKK3/6. In contrast to the results seen with a 6 h exposure to DBT, there was a very substantial increase in the phosphorylation of p44/42 at the 10 μ M concentration (4.6 fold) (Figure 5D and E). The immediate upstream activator of p44/42, MEK1/2, was activated 1.6 fold after a 6 h exposure to 10 μ M DBT (Figure 5D and F). Consistent with the 10 min and 60 min results, there were no significant changes in the phosphorylation of JNK or MKK4 following 6 h exposures of NK cells to 10-0.5 μ M DBT (data not shown).

Effect of DBT on MAPK phosphatase activity

Although we saw activation of MAP2Ks at every concentration of DBT that caused activation of MAPKs, it was also possible that increased phosphorylation of MAPKs could at least in part be due to DBT-induced decreases in MAPK phosphatase activity. Thus, lysates from NK cells treated with 10-0.5 μ M DBT for 10 min at 37°C were used to measure the activity of ser/thr phosphatase activity. MAPK phosphatases are ser/thr phosphatases so this assay indicated whether DBT exposures were able to decrease MAPK phosphatase activity which could have also accounted for the observed increases in MAPK phosphorylation. The results indicate that there was no significant difference among the treatments as compared to control (data not shown). Thus, these results indicate that DBT exposure is not inhibiting MAPK phosphatase activity.

DISCUSSION

We have previously shown that DBT has the ability to significantly disrupt human NK cell lytic function, *in vitro* (Whalen *et al.*, 1999, 2002, Dudimah *et al.*, 2007). It was evident in those studies that a 1 h exposure to as little as 0.5 μ M DBT induced permanent and progressive loss of NK lytic ability when the cells were placed in DBT-free media for as long as 6 days. Levels of DBT in human blood have been found to range from undetectable to as high as 0.31 μ M (Kannan *et al.*, 1999; Whalen *et al.*, 1999). Our previous studies have also shown that there is a loss of cell surface protein expression and of cytolytic proteins (granzyme B and perforin) that accompany this loss of lytic function (Odman-Ghazi *et al.* 2003; Catlin *et al.*, 2005). In this study we examine the effects of DBT exposures on several enzymes whose functions are key to regulating NK cell function, in an effort to elucidate the mechanism by which DBT causes NK cells to lose function. We have shown that another butyltin compound, TBT, which also decreases NK function, causes alterations of some of these key enzymatic activities (Aluoch and Whalen, 2005; Aluoch *et al.*, 2006; Aluoch *et al.* 2007). Specifically, MAPKs and MAP2Ks were activated by TBT exposures. MAPKs are regulators of the lytic response of NK cells (Vivier *et al.* 2004). Activation of these enzymes by TBT leaves the NK cell incapable of responding to an appropriate target cell, due to the fact that the MAPKs have been previously activated and are unavailable for activation by the target (Dudimah *et al.*, 2009).

In this study we found that 10 min exposures to DBT caused activation of the MAPK, p38, at every concentration tested. This activation was accompanied by an activation of the enzyme that activates p38, the MAP2K, MKK3/6. This same length of exposure to DBT did not activate

the MAPK, JNK. However, it did activate p44/42 and its MAP2K, MEK1/2, but only at the two highest concentrations of DBT. After 1 h there remained a significant increases in phosphorylation of p38 and MKK3/6 at all but the lowest DBT concentration, but there was no longer a significant activation of p44/42. The DBT-induced activations of p38 and MKK3/6 maintained out to 6 h, but they were only significant at the 10 μ M concentration. Interestingly, after a 6 h exposure to 10 μ M DBT there was a very significant activation of p44/42. Thus, the ability of DBT to activate p38 diminished with length of exposure, which was similar to what was seen when NK cells were exposed to TBT (Aluoch et al., 2006). Unlike p38, the activation of p44/42 by DBT was greatest at a 6 h exposure. p38 appears to be the most sensitive of the MAPKs to DBT exposures, as even the 0.5 μ M concentration caused increased phosphorylation of p38 at 10 min of exposure. This result is in contrast to that seen with TBT exposures, where p44/42 was the most sensitive MAPK (Aluoch et al. 2006)

The activations of MAP2K, MKK3/6 and MEK1/2 due to a 10 min exposure to DBT suggested an involvement of either MAP3Ks or MAP2K phosphatases. To begin to elucidate how DBT activates MKK3/6 and MEK1/2, we have assessed DBT effect(s) on the most upstream signaling proteins in this pathway, the PTKs. NK cell exposure to DBT for 10 min did not significantly activate ZAP-70, Syk, Src or PYK2. Thus, DBT-induced activation of either MKK3/6 or MEK1/2 does not appear to be a result of PTK activation leading to MAP3K activation. However, the accumulation of the phosphorylated MKK3/6 and MEK1/2 may be due to direct DBT-induced activation of the immediate upstream activator of the MAP2Ks, MAP3Ks (such as Raf-1) (Pearson *et al.*, 2001) and/or DBT-induced inhibition of the enzymes responsible for dephosphorylating the MAP2Ks, MAP2K phosphatases (Camps *et al.*, 2000; Mao *et al.*, 2005). Physiologically, target cell binding inactivates NK cells with persistent parallel loss of phosphatase activity (Bajpai and Brahmi, 1994). Pharmacological Ser/Thr phosphatase inhibitors also inhibit cytotoxic function (Bajpai and Brahmi, 1994). Inhibition of protein phosphatases, which inactivate MAPKs and MAP2Ks (Gomez and Cohen, 1991; Anderson *et al.*, 1990; Ishihara *et al.*, 1989) will lead to activation of MAP2Ks and MAPKs. We addressed this possibility using a phosphatase assay and found that there was no DBT-induced alteration of phosphatase activity. This suggests that the increase in MAP2K and MAPK phosphorylation seen with DBT are not due to inhibition of phosphatase activity and thus may be due to direct DBT-induced activation of MAP3Ks. Studies examining the ability of DBT to activate MAP3Ks (Rafs, ASK-1) using direct assay of MAP3K kinase activity are underway.

This study has widened our understanding of how an initial exposure to a range of DBT concentrations can cause inhibition of cytotoxic function: (1) DBT (10-0.5 μ M) can significantly activate both p38 and p44/42 within 10 min of exposure; (2) activation of p38 by 10-0.5 μ M DBT can be accounted for by DBT-induced activation of its upstream activator MKK3/6; (3) activation of p44/42 by 10 and 5 μ M DBT can be accounted for by DBT-induced activation of its upstream activator MEK1/2; (4) activation of p38 and MKK3/6 by 0.5 μ M DBT lasts less than 1 h while their activation by 10-1 μ M DBT can last for at least 1 h, only the activation seen at 10 μ M DBT maintains for 6 h; (5) activation of p44/42 and MEK1/2 by 10 and 5 μ M DBT lasts less than 1 h while their activation by 10 μ M DBT returns by 6 h; (6) at all concentrations of DBT where MAPKs were activated, inhibition of cytotoxic function was also seen (Dudimah et al, 2007) ; (7) DBT-induced activation of MAPKs and MAP2Ks is not due to DBT-induced activation of the most upstream signaling proteins in this pathway, PTKs.

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REFERENCES

- Aluoch AO, Odman-Ghazi SO, Whalen MM. Pattern of MAP kinases p44/42 and JNK activation by non-lethal doses of tributyltin in human natural killer cells. *Arch. Toxicol* 2007;81:271–277. [PubMed: 17019560]
- Aluoch AO, Odman-Ghazi SO, Whalen MM. Alteration of an essential NK cell signaling pathway by low doses of tributyltin in human natural killer cells. *Toxicology* 2006;224:229–237. [PubMed: 16781040]
- Aluoch AO, Whalen MM. Tributyltin-induced effects on MAP kinases p38 and p44/42 in human natural killer cells. *Toxicology* 2005;209:263–277. [PubMed: 15795062]
- Anderson NG, Maller JL, Tonks NK, Sturgill TW. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* 1990;343:651–653. [PubMed: 2154696]
- Bajpai A, Brahmi Z. Target cell-induced inactivation of cytolytic lymphocytes. Role and regulation of CD45 and calyculin A-inhibited phosphatase in response to interleukin-2. *J Biol Chem* 1994;269:18864–18869. [PubMed: 8034641]
- Biron CA. Activation and function of natural killer cell responses during viral infections. *Curr. Opin. Immunol* 1997;9:24–34. [PubMed: 9039782]
- Camps M, Nichols A, Arkinstall S. Dual Specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J* 2000;14:6–16. [PubMed: 10627275]
- Catlin R, Shah H, Bankhurst AD, Whalen MM. Dibutyltin exposure decreases granzyme B and perforin in human natural killer cells. *Environmental Toxicology and Pharmacology* 2005;20:395–403.
- Chan G, Hanks T, Fisher K-D. Vav-1 regulates NK T cell development and NK cell cytotoxicity. *Eur. J. Immunol* 2001;31:2403–2410. [PubMed: 11500824]
- Derijard B, Raingeaud J, Barret T, Wu IH, Han J, Ulevitch RJ, Davis RJ. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* 1995;267:682–685. [PubMed: 7839144]
- Djeu JY, Jiang K, Wei S. A view to a kill: signals triggering cytotoxicity. *Clin. Cancer Res* 2002;8:636–640. [PubMed: 11895890]
- Dudimah FD, Griffey D, Wang X, Whalen MM. Activation of p44/42 MAPK plays a role in the TBT-induced loss of human natural killer (NK) cell function. *Cell Biology and Toxicology*. 2009 in press NIHMSID#138216.
- Dudimah FD, Gibson C, Whalen MM. Effect of Dibutyltin (DBT) on ATP levels in human natural killer cells. *Environ. Toxicol* 2007;22:117–123. [PubMed: 17366562]
- Ema M, Iwase T, Iwase Y, Oyama N, Ogawa Y. Change of embryotoxic susceptibility to di-n-butyltin dichloride in cultured rat embryos. *Arch. Toxicol* 1996;70:742–748. [PubMed: 8896720]
- Epstein RL, Phillippo ET, Harr R, Koscinski W, Vosco G. Organotin residue determination in poultry and turkey sample survey in the United States. *J. Agric. Food Chem* 1991;39:917–921.
- Forsyth DS, Weber D, Barlow L. The determination of organotin compounds in fruit juices using gas chromatography-atomic absorption spectrometry. *Appl. Organomet. Chem* 1992a;6:579–585.
- Forsyth DS, Weber D, Clidroux C. Determination of butyltin, cyclohexyltin and phenyltin compounds in beers and wines. *Food Addit. Contam* 1992b;9:161–169. [PubMed: 1499773]
- Gismondi A, Jacobelli J, Mainiero F, Paolini R, Piccoli M, Frati L, Santoni A. Cutting Edge: Functional Role for Proline-Rich Tyrosine Kinase 2 in NK Cell-Mediated Natural Cytotoxicity. *J. Immunol* 2000;164:2272–2276. [PubMed: 10679059]
- Gomez N, Cohen P. Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. *Nature* 1991;353:170–173. [PubMed: 1716348]
- Han J, Lee JD, Jiang Y, Li Z, Feng L, Ulevitch RJ. Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J Biol. Chem* 1996;271:2886–2891. [PubMed: 8621675]
- Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem. Biophys. Res. Commun* 1989;159:871–877. [PubMed: 2539153]
- Jewett A, Bonavida B. Target induced anergy of natural killer cytotoxic function is restricted to the NK-target conjugate subset. *Cell. Immunol* 1995;160:91–97. [PubMed: 7842490]

- Kannan K, Senthilkumar K, Giesy JP. Occurrence of butyltin compounds in human blood. *Environ. Sci. Technol* 1999;33:1776–1779.
- Kannan K, Guruge KS, Thomas NJ, Tanabe S, Giesy JP. Butyltin residues in southern sea urchins (*Enhydra lutris nereis*) found dead near California coastal waters. *Environ. Sci. Technol* 1998;32:1169–1175.
- Kannan K, Falandysz J. Butyltin residues in sediment, fish, fish-eating birds harbor porpoise and human tissues from the Polish coast of the Baltic Sea. *Mar. Pollut. Bulletin* 1997;34:203–207.
- Mao L, Yang L, Arora A, Choe ES, Zhang G, Liu Z, Fibuch EE, Wang JQ. Role of protein phosphatase 2A in mGluR5-regulated MEK/ERK phosphorylation in neurons. *J. Biol. Chem* 2005;280:12602–12610. [PubMed: 15661743]
- Merkord J, Jonas L, Weber H, Kroning G, Nizze H. Acute interstitial pancreatitis in rats induced by dibutyltin dichloride (DBDC) pathogenesis and natural cause of lesions. *Pancreas* 1997;15:392–401. [PubMed: 9361094]
- Nakashima H, Hori S, Nakazawa H. Determination of dibutyltin and dioctyltin compounds in PVC food containers, wrappage and clothes by reversed phase HPLC with column switching. *Eisei Kagaku* 1990;36:155–220.
- Nielsen JB, Strand J. Hepatic deposition of butyltin in humans. *Metal Ions in Biol. and Med* 2002;7:185–188.
- Noda T, Morita S, Bab A. Teratogenic effects of various di-n-butyltins with different anions and butyl (3-hydroxyl) tin dilaurate in rats. *Toxicology* 1993;85:149–160. [PubMed: 8303710]
- Odman-Ghazi SO, Hatcher F, Whalen MM. Expression of Functionally Relevant Cell Surface Markers in Dibutyltin-exposed Human Natural Killer Cells. *Chemico-Biological Interactions* 2003;146:1–18. [PubMed: 12902148]
- Payne DM, Rossomando AJ, Martino P, Erickson AK, Her JH, Shabanowitz J, Hunt DF, Weber MJ, Sturgill TW. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO Journal* 1991;10:885–892. [PubMed: 1849075]
- Pearson G, Robinson F, Beers Gibson T, Xu B-E, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocrine Rev* 2001;22:153–183. [PubMed: 11294822]
- Pieters RH, Bol M, Seinen W, Penninks AH. Cellular and molecular aspects of organotin-induced thymus atrophy. *Hum. Exp. Toxicol* 1994;12:876–879. [PubMed: 7718308]
- Roper WL. “Toxicological profile for tin”. U.S department of health and human services, agency for toxic substances and disease registry. 1992
- Sadiki A-I, Williams DT, Carrier R, Thomas B. Pilot study on the contamination of drinking water by organotin compounds from PVC materials. *Chemosphere* 1996;32:2389–2398. [PubMed: 8653382]
- Trinchieri G. Biology of Natural Killer cell. *Adv. Immunol* 1989;47:187–376. [PubMed: 2683611]
- Trotta R, Puorro KA, Paroli M, Azzoni L, Abebe B, Eisenlohr LC, Perussia B. Dependence of both spontaneous and antibody-dependent, granule exocytosis-mediated NK cell cytotoxicity on extracellular signal-regulated kinases. *J. Immunol* 1998;161:6648–6656. [PubMed: 9862693]
- Trotta R, Fettuciar K, Azzoni L, Abebe B, Puorro KA, Eisenlohr LC, Perussia B. Differential role of p38 and c-Jun N-terminal kinase 1 mitogen-activated protein kinases in NK cell cytotoxicity. *J. Immunol* 2000;165:1782–1789. [PubMed: 10925255]
- Vivier E, Nunes JA, Vely F. Natural killer cell signaling pathways. *Science* 2004;306:1517–1519. [PubMed: 15567854]
- Wei S, Gamero AM, Liu JH, Daulton AA, Valkov NI, Trapani JA, Larner AC, Weber MJ, Djeu JY. Control of lytic function by mitogen-activated protein kinase/extracellular regulatory kinase 2 (ERK2) in a human natural killer cell line: identification of perforin and granzyme B mobilization by functional ERK2. *J. Exp. Med* 1998;187:1753–1765. [PubMed: 9607917]
- Whalen MM, Loganathan BG, Kannan K. Immunotoxicity of environmentally relevant concentrations of butyltins on human natural killer cells *in vitro*. *Environ. Res* 1999;81:108–116. [PubMed: 10433842]
- Whalen MM, Green SA, Loganathan BG. Brief Butyltin Exposure Induces Irreversible Inhibition of the Cytotoxic Function on Human Natural Killer Cells, *In vitro*. *Environ Res* 2002;88:19–29. [PubMed: 11896664]

Yamada S, Fuji Y, Mikami E, Kawamura N, Hayakawa J. Small-scale survey of organotin compounds in household commodities. *J. AOAC. Int* 1993;76:436–441.

A.

B.

C.

Figure 1.

Effects of 10 min exposures to 10 to 0.5 μ M DBT on phospho-p38, and phospho-MKK3/6 in NK cells: (A) levels of phospho-p38 and phospho-MKK3/6 normalized to control in pure NK. Values are mean $\pm$ S.D. from at least three separate experiments using cells from different donors * indicates significant difference compared to the control ($p < 0.05$). (B) Representative experiment for phospho-p38. The density values (arbitrary units) for phospho-p38 in this representative blot were: control=26399; 10 μ M=52511; 5 μ M=56184; 2.5 μ M=48658; 1 μ M=54985; 0.5 μ M=35931. These values are raw values and have not been corrected for loading differences (by normalization to actin). (C) Representative experiment for phospho-

MKK3/6. The density values for phospho-MKK3/6 in this representative blot were:
control=48613; 10 μ M=72130; 5 μ M=83952; 2.5 μ M=54372; 1 μ M=53313; 0.5 μ M=43310.

A.

B.

C.

Figure 2.

Effects of 10 min exposures to 10 and 5 μM DBT on phospho-p44/42, and phospho-MEK1/2 in NK cells: (A) levels of phospho-p44/42 and phosph-MEK1/2 normalized to control. Values are mean $\pm$ S.D. from at least three separate experiments using cells from different donors* indicates significant difference compared to the control (p<0.05). (B) Representative experiment for phospho-p44/42. The density values (arbitrary units) for phospho-p44/42 in this representative blot were: control=11780; 10 μM=30551; 5μM=27244; 2.5 μM=26660; 1 μM=15345; 0.5 μM=16234. (C) Representative experiment for phospho-MEK1/2. The density values for phospho-MEK1/2 in this representative blot were: control=46842; 10 μM=85910; 5μM=80800; 2.5 μM=50317; 1 μM=74064; 0.5 μM=37678.

A. **B.** **C.** **Figure 3.**

Effects of 10 min exposures to 10 to 0.5 μM DBT on phosphorylation of JNK, MKK4, and Protein tyrosine kinases (Syk, ZAP-70, Pyk and Src) in NK cells. (A) Representative experiment of phospho-JNK. (B) Representative experiment of phospho-MKK4. (C) Representative experiment for phospho-Syk, phospho-ZAP-70, phospho-Pyk and phospho-Src.

Figure 4.

Effects of 60 min exposures to 10 to 0.5 μ M DBT on phospho-p38, phospho-MKK3/6, phospho-p44/42, and phospho-MEK1/2 in NK cells: (A) levels of phospho-p38 and phospho-MKK3/6 normalized to control* indicates significant difference compared to the control ($p < 0.05$). Values are mean $\pm$ S.D. from at least three separate experiments using cells from different donors. (B) Representative experiment for phospho-p38. (C) Representative experiment for phospho-MKK3/6. (D) Representative experiment for phospho-p44/42. (E) Representative experiment for phospho-MEK1/2.

A.

B.

C.

D.

E.

F.

Figure 5.

Effects of 6 hour exposures to 10 μM DBT on phospho-p38, phospho-MKK3/6, phospho-p44/42, and phospho-MEK1/2 in NK cells: (A) levels of phospho-p38 and phospho-MKK3/6 normalized to control* indicates significant difference compared to the control (p < 0.05) Values are mean ± S.D. from at least three separate experiments using cells from different donors. (B) Representative experiment for phospho-p38. (C) Representative experiment for phospho-MKK3/6. (D) levels of phospho-p44/42 and phospho-MEK1/2 normalized to control. Values are mean ± S.D. from at least three separate experiments using cells from different donors. (E) Representative experiment for phospho-p44/42. (F) Representative experiment for phospho-MEK1/2.

TABLE 1

Antibodies used in Western blot analysis

Antibody	Specificity
Anti-phospho-p38	Human p38 phosphorylated on T180 and Y182
Anti-p38	Human p38
Anti-phospho-p44/42	Human p44/42 phosphorylated on T202 and Y204
Anti-p44/42	Human p44/42
Anti-phospho-MKK3/6	Human MKK3/6 phosphorylated on S189 and S207
Anti-MKK3/6	Human MKK3/6
Anti-phospho-MEK1/2	Human MEK1/2 phosphorylated on S217/S221
Anti-MEK1/2	Human MEK1/2
Anti-phospho-Src (Tyr416)	Human Src phosphorylated on Y416
Anti-phospho-Pyk2 (Tyr402)	Human Pyk2 phosphorylated on Y402
Anti-phospho-Syk (Tyr525/526)	Human Syk phosphorylated on Y525 and Y526
Anti-phospho-Zap-70 (Tyr319),	Human Zap-70 phosphorylated on Y319
Anti- β -actin	Human β -actin

All antibodies were obtained from Cell Signaling Technologies, Beverly, MA