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Ugochukwu Nnodu
Tennessee State University

Margaret M. Whalen
Tennessee State University

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Pentachlorophenol decreases ATP levels in human natural killer cells

Ugochukwu Nnodu and **Margaret M. Whalen**
Department of Chemistry, Tennessee State University, Nashville, TN 37209

Abstract

Pentachlorophenol (PCP) is used as a wood preservative and is found in human blood and urine. PCP causes significant decreases in the tumor-killing (lytic) function of human natural killer (NK) cells, a critical immune defense. The current study examined the association between decreased lytic function and decreased ATP levels, as well as the ability of antioxidants (vitamin E and reduced glutathione) to prevent PCP-induced decreases in either ATP levels or lytic function. Exposure of NK cells to 10μM PCP decreased ATP levels by 15% at 24 h, and exposure to 5 μM PCP decreased ATP levels by 32% at 48 h. No effects were seen with 0.5 μM at 48 h or with 5 μM at 24 h. However, 10 μM PCP decreased lytic function by 69% at 24h and 5 μM decreased it by 90% at 48 h. Even 0.5 μM PCP decreased lytic function by 46% at 48 h. None of these effects were prevented by pretreatment with 1 mM vitamin E or reduced glutathione.

Keywords

NK cells; pentachlorophenol; lytic function; ATP levels; vitamin E; glutathione

INTRODUCTION

Pentachlorophenol (PCP) was at one time registered for use as an insecticide, fungicide, herbicide, molluscicide, algicide, disinfectant, and ingredient in antifouling paints (Cirelli 1978). Its use was restricted in 1984 (EPA, 2001). It is now mainly used as a wood preservative for power-line poles and fence posts. Approximately 80% of the PCP used in the U.S. is for the treatment of wooden utility poles (ATSDR, 2001). As with other organochlorine compounds PCP is quite stable. Due to its once widespread use and chemical stability, it is a very significant environmental contaminant. Humans are exposed to PCP through inhalation, absorption through the skin, and consumption of contaminated food and water (ATSDR, 2001). Measurable levels of PCP have been detected in human blood and urine (Atuma and Okor, 1985; CDC, 2005; Cline et al., 1989; Jones et al., 1986; Thornton et al., 2002; Uhl et al. 1986). Levels ranging from 0.26 −5 μM were found in the serum of individuals residing in PCP-treated log homes (Cline et al., 1989). Levels averaging about 0.15 μM were found in the serum of individuals with no known exposure (Cline et al., 1989; Uhl et al., 1986).

Natural Killer (NK) cells are a subset of lymphocytes that are capable of killing tumor cells, virally infected cells and antibody-coated cells. NK cells are capable of killing tumor cells without prior sensitization, putting them in the forefront of lymphocyte defense against tumor cells and virally infected cells (Vivier et al., 2004; Wu and Lanier, 2003). NK cells are defined by the absence of the T cell receptor/CD3 complex and by the presence of CD56 and/or CD16

*Corresponding author. Present address: Margaret M. Whalen, Ph. D. Department of Chemistry, Tennessee State University 3500 John A. Merritt Blvd., Nashville, TN 37209−1561, USA Phone: 615−963−5247 Fax: 615−963−5326 Email: mwhalen@tnstate.edu.

on the cell surface. About 95% of NK cells in human peripheral blood are $CD56^{dim}/CD16^{bright}$ and express high levels of the cytotoxic protein, perforin (Walzer et al. 2005). They are responsible for limiting the spread of blood borne metastases, as well as limiting the development of primary tumors (Hanna, 1980; Kiessling and Haller, 1978). NK cells also play a central role in immune defense against viral infection as evidenced by greatly increased incidence of viral infection seen in individuals where the NK subset of lymphocytes is completely absent (Fleisher et al., 1982; Biron et al, 1989). Therefore, any chemical that can affect the ability of NK cells to recognize and lyse a target cell, could potentially increase the risk of tumors and viral infections.

In a past study we found that exposure of human NK cells to PCP decreased their lytic function. Exposure of NK cells to as little as 0.5 μM PCP for 48 h decreased lytic function about 50% (Reed et al., 2004). The mechanism by which PCP exposure might be interfering with NK cell lytic function was not addressed in that study.

In this study we examined the effects of PCP exposures on ATP levels in NK cells in order to begin to elucidate the mechanism by which PCP decreases lytic function. This data will allow us to determine if PCP is able to interfere with ATP production in NK cells and whether any interference is associated with loss of NK lytic function. Additionally, we examined whether the antioxidants, vitamin E or reduced glutathione, were able to reverse the negative effects of PCP on lytic function and/or ATP levels, as there has been evidence in rodents (Dorsey et al., 2006; Umemura et al., 2006; 2003) and in human hepatoma cell lines (Wang et al., 2001) and human liver cell microsomes (Juhl et al., 1985) that PCP oxidative metabolism is important in its toxic effects in those systems.

MATERIALS AND METHODS

Isolation of NK cells

Peripheral blood from healthy volunteer donors (male and female adults) was used for this study. Fresh blood bank buffy coats were collected at a Red Cross donation facility (American Red Cross, Portland, OR). Highly purified NK cells were obtained using a rosetting procedure. Buffy coats were mixed with 1 mL of RosetteSep™ Human NK cell enrichment antibody cocktail (StemCell Technologies, Vancouver, British Columbia, Canada) per 30 mL of buffy coat. The mixture was incubated for 25 min at room temperature with periodic mixing. Following the incubation 4 mL of the mixture was layered onto 4 mL of Ficoll-Hypaque (1.077 g/mL) (Sigma), and centrifuged at 1200g for 30 min. The cell layer was collected and washed twice with PBS and stored in complete media (RPMI-1640 supplemented with 2 m*M L*-glutamine, 50 U penicillin G and 50 μg streptomycin/ml) plus 10% heat-activated bovine calf serum (BCS) at 1 million cells/mL. The resulting cell preparation was >95% $CD16^+$, 0% $CD3^+$ by fluorescence microscopy (Reed et al., 2004).

Chemical Preparation

PCP was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). A stock solution of PCP was made in DMSO. The stock was diluted in gelatin media (0.5% gelatin replaced the calf serum in complete medium) to achieve the desired concentrations, so that the final concentration of DMSO did not exceed 0.01%.

Cell Treatments and cell viability

NK cells were separated by centrifugation from complete medium (defined above) and transferred to complete medium containing 0.5% gelatin in place of the 10% bovine serum. This was done in order to avoid binding of the hydrophobic PCP to serum albumin, which could interfere with its delivery to the cells. NK cells were then exposed to PCP for 24 h, 48

h, or 6 days. The concentration range examined was 10 − 0.1 μM PCP depending on the length of exposure.

Cell viability was determined by trypan blue exclusion. Cell numbers and viability were assessed at the beginning and end of each exposure period. Cell numbers and their viability did not vary significantly among experimental conditions for any of the concentrations of the compounds used in the experiments at a given length of incubation.

Chromium Release Assay

NK cytotoxicity was measured using a ^{51}Cr release assay (Whalen et al., 1999; Whalen, 1997). The target cell in all cytotoxicity assays was the NK-susceptible K562 (human chronic myelogenous leukemia) cell line. K562 cells were incubated with ^{51}Cr in 1 ml of BCS for 1.5 hr at 37 °C in air/CO_2 (19:1). Following this incubation the target cells were washed twice with gelatin medium. NK (effector) cells (1.2×10^5/100 μL for 12:1 ratio) were added to the wells of round-bottomed microwell plates. The effectors were diluted to 6:1 ratio (0.6×10^5/100 μL) and 3:1 ratio (0.3×10^5/100 μL); each ratio was tested in triplicate. Target cells were added (1×10^4/100μL) to the wells and the plate was centrifuged at 300 g for 3 min and incubated for 2 hr at 37 °C (air/CO_2 ,19:1). After incubation a 0.1 ml aliquot of the supernatant was collected and counted for radioactivity for 60 sec in a Packard COBRA gamma radiation counter (Packard Instrument Co., Meriden, CT). Target lysis was calculated as follows: 100×[(test c.p.m - spontaneous c.p.m.)/maximum c.p.m.- spontaneous c.p.m.)]. Maximum release was produced by adding 100 μL of 10% Triton X-100. Statistical analysis of the data was carried out utilizing ANOVA and Student's t test. Data were initially compared within a given experimental setup by ANOVA. A significant ANOVA was followed by pair wise analysis of control versus exposed data at each NK to target ratio and each concentration using Student's t test.

ATP assay

ATP levels were measured as described by Dudimah et al., 2007a,b. Following the cell exposure to the various concentrations of PCP; 250,000 NK cells (167 μL of the cell suspension) were removed, diluted by addition of 300 μL of PBS, and centrifuged for ninety seconds to pellet the cells. All media was removed and the cell pellet was resuspended in 375 μL of distilled water. 150 μL of this suspension was added to 300 μL of somatic cell ATP releasing reagent (Sigma-Aldrich). To measure ATP, 100 μL of the lysed cell suspension was added to 100 μL of a Luciferin/Luciferase mixture. The light emission was measured using the Kodak Imaging system (Kodak, Rochester NY). ATP levels were determined from a standard curve. Statistical analysis of the data was carried out utilizing ANOVA and followed by pairwise comparison using Student's t test.

RESULTS

Effects of 24 h, 48 h, and 6 d exposures to PCP on ATP levels in NK cells

Figure 1 shows the effects of 24 h, 48 h, and 6 d exposures to 0.1 to 10 μM PCP on ATP levels in human NK cells. A 24 h exposure to 10 μM PCP caused an approximately 15% decrease in the level of ATP in NK cells as compared to control cells ($p<0.01$) (Fig. 1A). After a 48 h exposure to 5 μM PCP there was about a 32% decrease in ATP levels ($p<0.01$). A 48 h exposure to both 2.5 and 1 μM PCP caused small decreases in ATP levels (12−15%, $p<0.01$) (Fig. 1B). Exposure to PCP for 6 days caused decreases in ATP, at 1, 0.5 and 0.2 μM, of 67%, 35%, and 20% respectively ($p<0.01$) (Fig. 1C). The highest concentration used at any given time point did not affect NK cell viability.

Comparison of the effects of PCP on lytic function and ATP levels in NK cells

Although previous studies have shown decreased lytic function with PCP exposures (Reed et al., 2004), it was important to carry out studies of lytic function in parallel with those determining ATP levels. This will allow a valid comparison of these two parameters using cells prepared on the same day. The decreases in ATP at these same concentrations of PCP were plotted on the same graph to aid in seeing the extent of association between the two parameters.

Figure 1 shows the results of studies examining the lytic function of NK cells exposed to 0.5 μM to 10 μM PCP for 24 h, 48 h and 6 d. A 24 h exposure to PCP caused significant decreases in lytic function at all concentrations (Fig. 1A). The decreases were 15%, 31%, 42%, 45%, and 69% at 0.5, 1, 2.5, 5, and 10 μM PCP, respectively ($p<0.01$).

Figure 1B shows the lytic function plotted along with ATP levels of NK cells exposed to 0.5 − 5 μM PCP for 48 h. Lytic function was decreased very significantly at all concentrations of PCP tested. Decreases were 46, 70, 83 and 90 % at 0.5, 1, 2.5 and 5 μM, respectively ($p<0.001$).

The lytic function of NK cells exposed to 0.2−1 μM PCP for 6 days is shown in Figure 1C. Cells exposed to 0.2 μM PCP showed no significant decrease in lytic function ($p>0.05$). However, cells exposed to either 0.5 and 1 μM PCP showed very large decreases in lytic function. The decrease in lytic function was 73% ($p<0.0001$) in cells exposed to 0.5 μM PCP and 88% ($p<0.0001$) in those exposed to 1 μM PCP.

Effects of vitamin E and reduced glutathione on the PCP-induced decreases in ATP

The antioxidant vitamin E was tested for its ability to prevent the deleterious affects of PCP on ATP levels in NK cells. NK cells were pre-incubated with 1 mM vitamin E for 1 h prior to the addition of PCP. The 1 mM vitamin E remained for the duration of the exposures to PCP. Figure 2 shows that when NK cell were exposed to 10 μM PCP for 24 h that the decrease in ATP levels of about 15% was not statistically changed by the presence of 1 mM vitamin E. Additionally, vitamin E was ineffective in preventing the approximately 32% decrease in ATP caused by a 48 h exposure to 5 μM PCP.

NK cells were also pre-incubated with 1 mM reduced glutathione (GSH) for 1 h prior to the addition of PCP, which remained for the duration of the exposures to PCP. Figure 2 shows that the presence of GSH did not diminish the decreases in ATP induced by PCP. The effect of PCP was in fact somewhat greater when GSH was present than in its absence ($P<0.05$).

Effects of vitamin E and reduced glutathione on the PCP induced decreases in lytic function

As seen in Figure 1 loss of lytic function by NK cells occurs prior to any decreases in ATP levels. Thus, we examined whether the antioxidants vitamin E or reduced glutathione might be able to prevent the loss of lytic function. NK cells were pre-incubated for 1 h prior to exposure to PCP with either 1 mM vitamin E or 1 mM reduced glutathione, which remained throughout the exposure to PCP. Figure 3 shows the results of studies examining 24 h exposure of NK cells to PCP concentrations from 0.5 − 5 μM plus and minus 1 mM vitamin E or 1 mM glutathione. There was no difference in effectiveness of the PCP to decrease NK lytic function whether or not the antioxidant was present at any of the PCP concentrations tested.

Vitamin E was also tested for its ability to prevent PCP-induced loss of lytic function after a 48 h or 6 d exposure PCP. As was seen after a 24 h exposure there was no change in the effectiveness of any concentration of PCP when vitamin E was present in the incubation (data not shown).

DISCUSSION

Our previous studies demonstrated that PCP was capable of interfering with the function of human NK cells (Reed et al., 2004). This PCP-induced decrease in NK function may play a role in the development of cancers that are associated with PCP exposures (Demers et al., 2006). It has been shown that PCP (~20 μM) can block oxidative phosphorylation in rat liver (Weinbach, 1954; Weinbach and Garbus, 1965), which would lead to decreases in ATP levels. In this study we found that exposure to PCP can decrease ATP levels in NK cells at higher concentrations and longer exposures (6 days). However, PCP decreased the lytic function of NK cells at concentrations that were lower than those that decreased ATP levels at 24 or 48 h of exposure. These results suggest that decreases in intracellular ATP levels are not an underlying cause of the losses of lytic function that are seen when NK cells are exposed to PCP for 24 h or 48 h.

A number of previous studies have shown that oxidative metabolites of PCP are responsible for some of its toxic effects in rodents (Dorsey et al., 2006; Umemura et al., 2006; 2003; Wang et al., 2001). Additionally, PCP has effects similar to butyltin (BT) compounds on NK lytic function and ATP levels (Whalen et al. 1999; Dudimah et al 2007a,b) and glutathione and other sulfhydryl containing compounds have been shown to reverse the negative effects of BT compounds on ATP (Cain et al., 1977). Thus, we examined the ability of the antioxidants, reduced glutathione and vitamin E, to ameliorate the negative effects of PCP exposures on NK lytic function and ATP levels. We found that neither of the antioxidants was able to prevent the decreases in lytic function or ATP levels seen when NK cells were exposed to PCP. This indicated that the oxidative metabolism of PCP does not significantly contribute to its toxic effects in human NK cells.

In summary these studies indicate that : 1.) ATP levels in NK cells are decreased by certain PCP exposures 2.) loss of NK lytic function seen with PCP exposure is not primarily due to decreases in ATP levels; 3.) losses of lytic function and decreases in ATP seen with PCP exposures do not appear to be the result of generation of reactive oxygen species or of the oxidative metabolism of PCP in the NK cell.

ACKNOWLEDGMENTS

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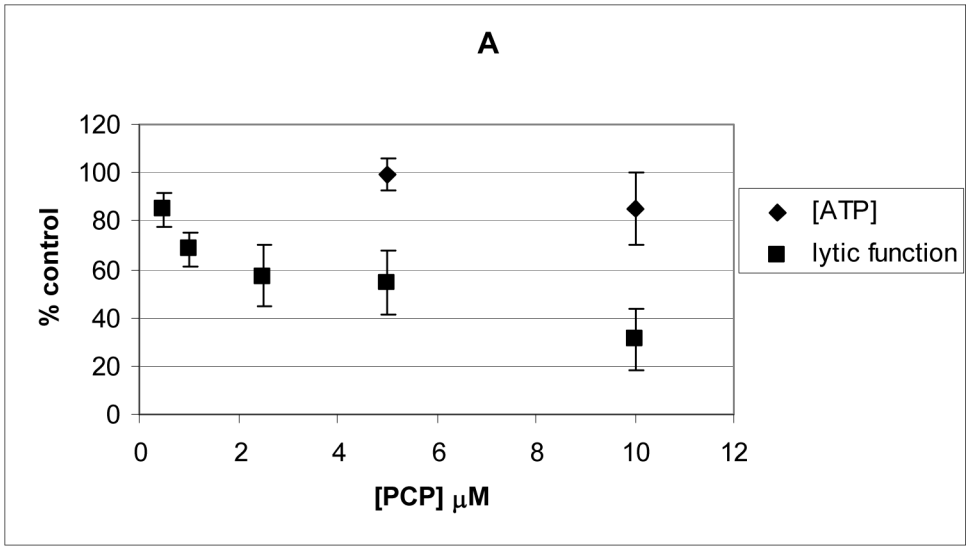

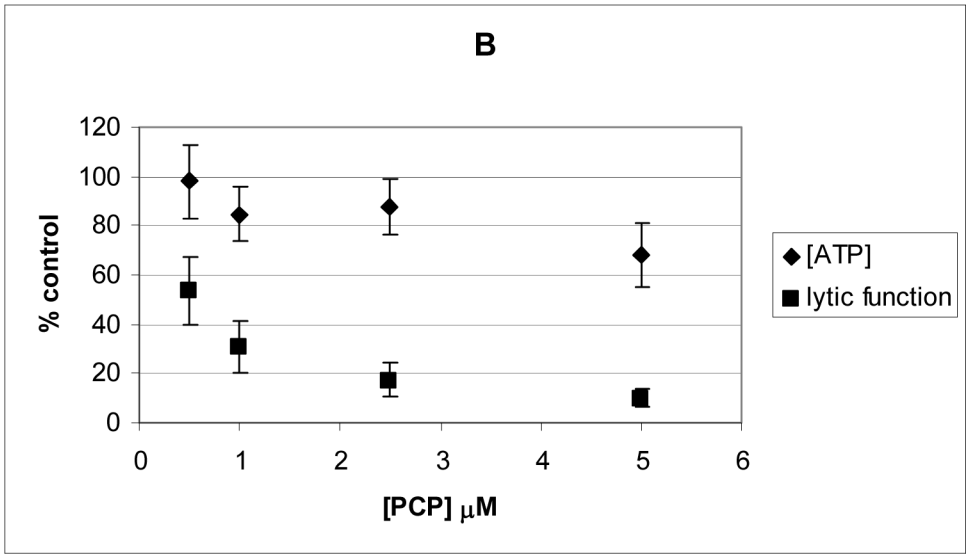

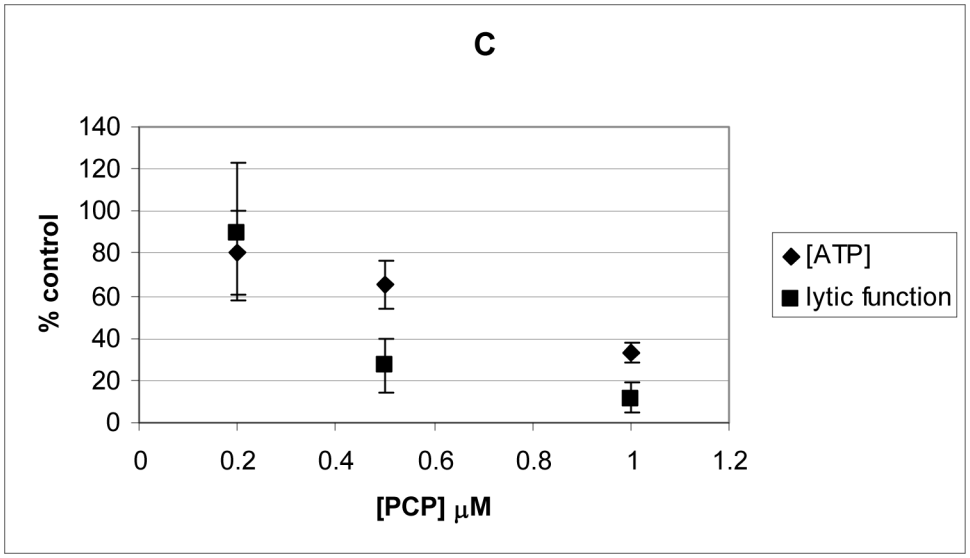

Figure 1.
Effects of exposures to Pentacholophenol (PCP) on ATP levels (▲) and lytic function (■) plotted on the same graph: A). 24 h exposure to PCP; B). 48 h exposure to PCP; and C). 6 day exposure to PCP. Levels of ATP in PCP exposed cells were determined as the percentage of ATP levels of the control cells. To combine results from separate experiments, the levels of lysis were normalized as percentage of the lytic function of the control cells in a given experiment. Results were the average ± S.D. of 9 or more determinations.

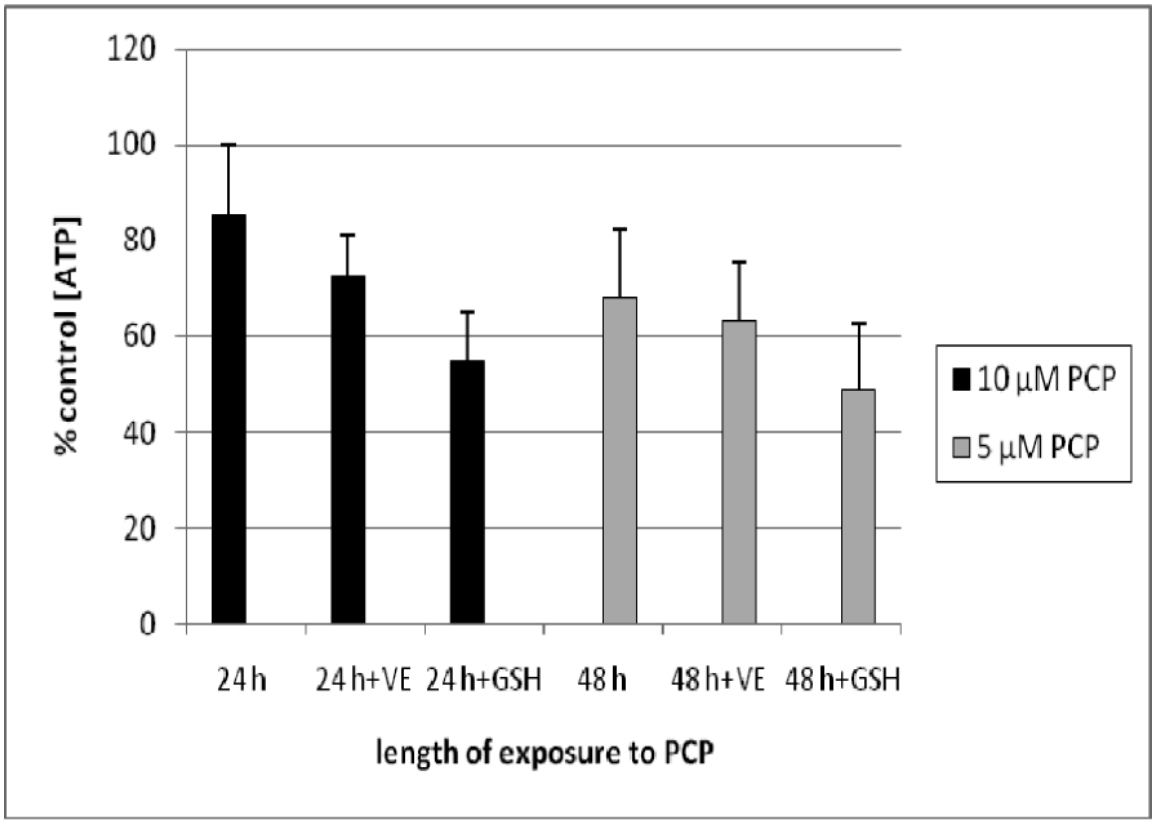

Figure 2.

Effects of a 1 h pre-incubation with 1 mM vitamin E or 1 mM reduced glutathione on the capacity of PCP to decrease ATP levels in NK cells. ATP levels were determined in NK cells that were pre-incubated with 1 mM Vitamin E (VE), 1 mM reduced glutathione (GSH) or control for 1 h prior to a 24 h exposure to 10 μM PCP or a 48 h exposure to 5 μM PCP. Results were the average ± S.D. of 9 or more determinations

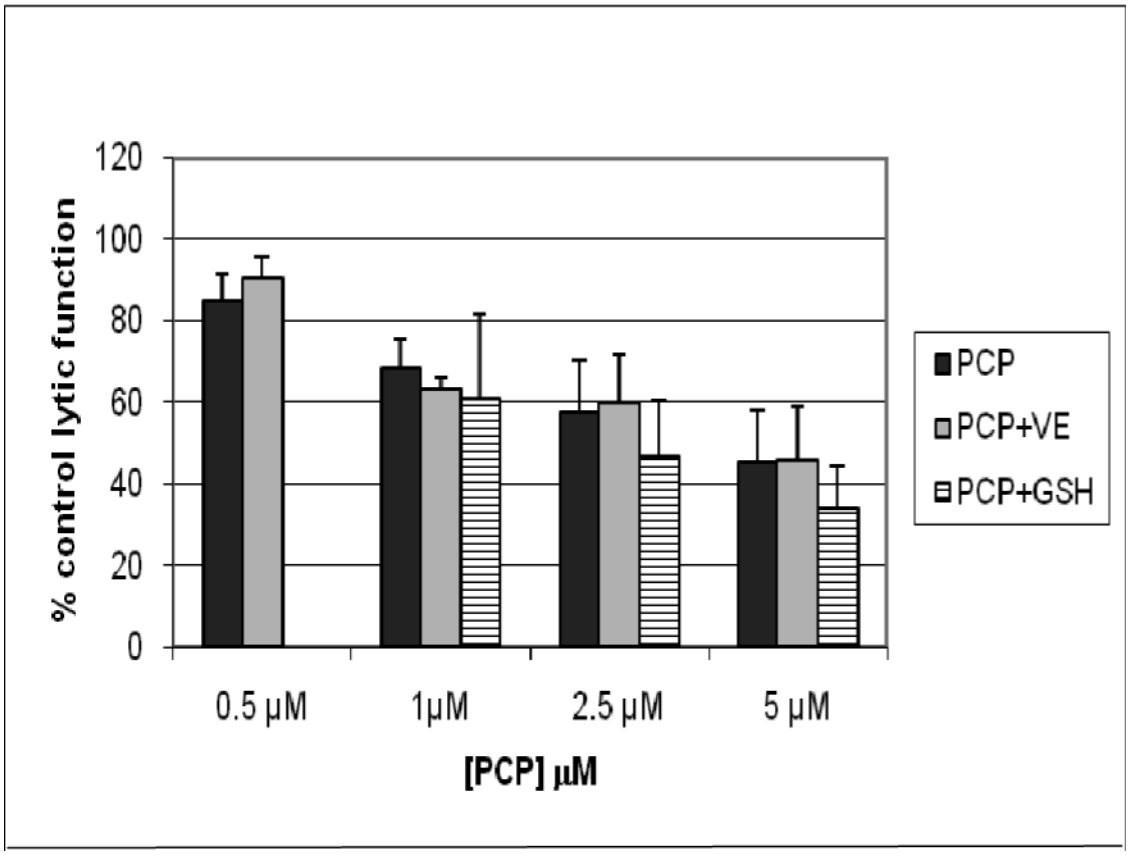

Figure 3.
Effects of a 1 h pre-incubation with 1 mM vitamin E or 1 mM reduced glutathione on the capacity of PCP to decrease the lytic function of NK cells. Lytic function was determined for NK cells that were pre-incubated with 1 mM Vitamin E (VE); 1 mM reduced glutathione (GSH), or control for 1 h prior to a 24 h exposure to 5−0.5 µM PCP. Results were the average ± S.D. of 6 or more determinations