Phenylarsine Oxide and Ethanol Prevent Cell Death of Porcine Polymorphonuclear Leucocytes Induced by Phorbol Myristate Acetate

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ABSTRACT. In this study, we report that phenylarsine oxide and ethanol, both of which suppress a number of polymorphonuclear leucocyte functions including superoxide production, prevented the phorbol myristate acetate-induced cell death in a dose-dependent manner. These reagents had an inhibitory effect even after polymorphonuclear leucocytes were stimulated to produce superoxide by treatment with phorbol myristate acetate. The results indicate that activation of protein kinase C and subsequent superoxide release do not directly cause phorbol myristate acetate-induced cell death. Phenylarsine oxide or ethanol prevents cell death by affecting pathways downstream from those involved in the superoxide production.

Polymorphonuclear leucocytes (PMNs) from mammalian peripheral blood are terminally differentiated cells that spontaneously undergo apoptosis in vivo and in vitro. A protein kinase C (PKC) activator, phorbol myristate acetate (PMA), previously was found to induce cell death of porcine PMNs accompanied by morphological degeneration within 3 to 5 hours in non-apoptotic fashion (1). Because another PKC activator, dioctanoyl glycerol, did not induce the cell death despite the fact that it stimulated superoxide production in a manner similar to PMA (1), it appears unlikely that death is a direct result of PKC activation and subsequent superoxide production. In this study, we examined the effects of phenylarsine oxide (PAO) and ethanol, both of which inhibit a number of PMN functions including superoxide generation (2-7), on the PMA-induced cell death. Both PAO and ethanol prevented the cell death when cells were exposed to these agents before or after the onset of superoxide production.

MATERIALS AND METHODS

Materials
PMA, MTT, cytochrome c, and PAO were purchased from Sigma Chemical Co., USA. PMA (500 µg/ml) and PAO (25 mM) were dissolved in DMSO and stored at −20°C. Ethanol was from Kanto Chemical Co., Japan. Glutaraldehyde and OsO4 were from TAAB, UK. Propylene oxide, Quetol-651, NSA and DMP-30 were from Nissin EM Co., Japan. All other chemicals were of analytical grade.

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Methods of procedure
PMNs were isolated from porcine peripheral blood as described (1). Three × 10⁶ cell/ml PMNs were incubated in phosphate-buffered saline containing 5 mM glucose (PBSG) with or without each agent at 37°C. PMN viability was assessed by the MTT assay, and superoxide production was measured as the rate of superoxide dismutase-inhibitable reduction of cytochrome c as described (1).

Transmission electron microscopy
Cells were fixed in 2.5% glutaraldehyde for 1 hour at room temperature and washed with 0.2 M phosphate buffer three times. Cells were postfixed in 1% OsO₄ for 30 min at 4°C and washed three times. Then they were dehydrated in ethanol and propylene oxide and embedded in Epoxy-resin (Quetol-651), which was polymerized at 60°C. Ultrathin sections were cut and stained with lead citrate and uranyl acetate prior to examination in a transmission electron microscope.

RESULTS AND DISCUSSION
PMA is known to stimulate various PMN functions including superoxide generation by activation of PKC (2). PMA induces non-apoptotic cell death of PMNs concomitant with morphological degeneration within 3 to 5 hours of exposure, an effect that is probably not due to toxicity of reactive oxygen species produced by PMA-activated PMNs because it is not inhibited by antioxidants and cell death does not occur after treatment with dioctanoyl glycerol, which also stimulates superoxide release (1). Therefore, we investigated whether inhibitors of PMN activation were able to suppress PMA-induced cell death.
Recently, phenylarsine oxide (PAO), an arsenical compound that reacts with vicinal thiols (8-11), has been found to inhibit PMN superoxide production independently of activation pathways (2, 10, 11). The molecular target of PAO remains to be identified, however. Pretreatment of PMA-stimulated PMNs with PAO inhibited superoxide generation in a dose-dependent manner, and doses higher than 2 μM PAO inhibited superoxide generation completely (Fig. 1). Next, we examined whether PAO prevented the PMA-induced cell death. In the absence of PMA, pretreatment with 2.5 μM PAO caused a high proportion of cells to die. At higher PAO concentrations (5 to 10 μM), however, death did not occur, although the MTT assay showed that absorbance values were lower than controls (Fig. 2a). Lower absorbances observed at 5 to 10 μM PAO were not due to a decrease in viability (trypan blue test, data not shown), possibly because metabolic activity of the cells was lower than controls. This unique dose response is consistent with a previous report that small changes in PAO concentration have opposite effects on human granulocyte survival (12), although the dose ranges for survival and death were differ from the present study.

When PMA (100 ng/ml) was added after pretreatment with various concentrations of PAO, a similar dose response curve was obtained (Fig. 2b). PMA induced cell death in the absence of PAO consistent with our previous report (1). While 2.5 μM PAO did not suppress death, higher concentrations (5 to 10 μM) were significantly inhibitory (Fig. 2a). Yousefi et al. (12) reported that PAO concentrations that blocked human granulocyte apoptosis also increased tyrosine phosphorylation. Lower PAO concentrations that accelerated apoptosis were associated with low levels of tyrosine phosphorylation, suggesting that granulocyte apoptosis is regulated in part by tyrosine phosphorylation. We also found that 5 to 10 μM PAO inhibited spontaneous DNA ladder formation seen in control cells (data not shown). Kutsumi et al. (10) also reported that PAO inhibited phosphotyrosine phosphatase activity, resulting in enhanced tyrosine phosphorylation in PMA-acti-

![Fig. 1](image1.png)  
**Fig. 1.** Inhibition of PMN superoxide production by PAO. Three \times 10^6 cell/ml were stimulated by 100 ng/ml PMA in the presence or absence of PAO. Reduction of cytochrome c by superoxides was measured as an increase in absorbance at 550 nm. One experiment representative of three is shown.

![Fig. 2](image2.png)  
**Fig. 2.** Effect of PAO on PMA-induced cell death assessed by the MTT assay. (a) PMNs were preincubated with PAO at the concentrations of 0, 2.5, 5, 10, 20 and 30 μM for 10 min. Then PMNs were incubated for 5 hours in the presence (open squares) or absence (open circles) of 100 ng/ml PMA. (b) 10 μM PAO was added at 0 min (PAO-P), 30 min (P30-PAO), 40 min (P40-PAO), 50 min (P50-PAO), 60 min (P60-PAO), 120 min (P120-PAO) and 180 min (P180-PAO) after treatment with PMA. PMNs were also incubated in the absence (CONTROL) or presence of PAO (PAO) or PMA (PMA) alone. All incubation times were 5 hours in total. Absorbance at 570 nm minus 630 nm was regarded as cell viability. Standard error bars (n=3) were within the symbols.
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vated human neutrophils. The results shown in Fig. 2a suggest that PAO may prevent PMA-induced cell death by a mechanism involving tyrosine phosphorylation, although PMA-induced cell death was non-apoptotic (1). However, PAO inhibited PMN superoxide production (Fig. 1) and this inhibition was independent of tyrosine phosphorylation (2). Thus it is possible that PAO suppresses PMA-induced cell death by inhibition of superoxide production.

Next we tested whether PAO could suppress cell death after PMA-treated PMNs had begun superoxide production (Fig. 2b). When cells were exposed to PMA and PAO simultaneously, death was prevented, but absorbance of the MTT assay rose when PAO was added after cells were preincubated with PMA for 30 min. At longer preincubation times viability decreased time dependently. PMA rapidly stimulates neutrophil superoxide generation and membrane vesiculation (13). In this study, PMN superoxide generation began at 7 to 8 min and ended 15 to 20 min after PMA treatment (data not shown). These results suggest that PAO inhibits PMA-induced cell death by a mechanism other than inhibition of superoxide generation. PAO is known to inactivate neutrophil cytosolic PKC, and this may be reversed by 2,3-dimercaptopropanol (10). PKC also may be inactivated by masking of vicinal thiols in its catalytic domain (14). Whether PKC inactivation is involved in suppression of PMA-induced cell death by PAO is currently unknown. However, it is possible that PAO prevents PKC down-regulation following activation by long-term exposure of cells to PMA (1).

Ethanol is well known as an inhibitor of some neutrophil functions (4-7), and neutrophil-mediated host defenses may be compromised in alcohol abusers (5, 6). Ethanol inhibits production of phosphatidic acid and diacylglycerol by phospholipase D (4, 7), and compromising neutrophil superoxide production (4, 6). This suggests that phospholipase D activation is functionally linked to superoxide generation (4). Dose-dependent ethanol inhibition of superoxide generation by PMA-stimulated PMNs also was observed in the present study (data not shown). Olson et al. (15) reported phospholipase D activation by PMA in a cell free system established from human neutrophils, and this was mediated via a PKC activating pathway. In human neutrophils stimulated by formyl Met-Leu-Phe, sustained diacylglycerol formation occurs predominantly by sequential action of phospholipase D and phosphatidic acid phosphohydrolase on phosphatidylcholine (16). Therefore, we tested whether ethanol suppresses PMA-induced cell death (Fig. 3). As shown in Fig. 3a, in the absence of PMA, exposure to 1 or 2% ethanol had no effect on the absorbance in the MTT assay and 3% ethanol moderately reduced the absorbance unaccompanied by cell death (trypan blue test; data not shown). At higher ethanol concentrations, viability decreased significantly. When PMA was added in the presence of ethanol, cell death was prevented (Fig. 3a), and while there were slight differences among experiments, ethanol was effective over a broad range of concentrations. Ethanol also had a suppressive effect on PMA-induced cell death when added 10–20 minutes after activation by PMA (Fig. 3b).

Morphologically, PMA-treated cells (Fig. 4b) showed characteristic severe degeneration as compared to control cells (Fig. 4). A significant increase in cell volume and a decrease in number of PMN granules were evident. Both PAO and ethanol inhibited these PMA-induced events (Fig. 4c and d).

Fig. 3. Effect of ethanol on PMA-induced cell death assessed by the MTT assay. (a), PMNs were incubated with ethanol at the concentrations of 0, 1, 2, 3, 4, 5, 6 and 7% in the presence (open squares) or absence (open circles) of 100 ng/ml PMA for 5 hours. (b), 3% ethanol was added at 0 min (E-P), 10 min (P10-E), 20 min (P20-E), 30 min (P30-E), 40 min (P40-E), 50 min (P50-E) and 60 min (P60-E) after treatment with PMA. PMNs were also incubated in the absence (CONTROL) or presence of ethanol (E) or PMA (P) alone. All incubation times were 5 hours in total. Standard error bars (n=3) were within the symbols.
that PMA, but not diacylglycerol, is a long-term activator of PKC that also causes PKC down-regulation, so PMA induces cell death possibly by toxic hyperactivation of PMNs. PAO and ethanol may inhibit such activation.

REFERENCES


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