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Inhibition of Human Platelet Phospholipase A_2 by Mono(2-ethylhexyl)phthalate

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There is evidence that the carcinogenic and teratogenic effects attributed to the plasticizer di(2-ethylhexyl)phthalate (DEHP) are due to its major metabolite mono(2-ethylhexyl)phthalate (MEHP). MEHP is also formed *ex vivo* by a plasma enzyme in blood products stored in polyvinyl chloride (PVC) DEHP plastic containers. People who receive large amounts of blood products, such as hemophiliacs or patients undergoing hemodialysis, cardiopulmonary bypass, or massive transfusion, are exposed to significant levels of plasticizer. In this study, the platelet was used to show that MEHP inhibits phospholipase A_2 (PLA_2), one of enzymes important in the release of arachidonic acid from membrane phospholipids. Arachidonate is the parent molecule for the synthesis of prostaglandins, thromboxanes, leukotrienes, and lipoxins that are made by a wide variety of cells. PLA_2 was measured by the liberation of ^{14}C-arachidonic acid from 1-stearoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylcholine. MEHP inhibits PLA_2 activity noncompetitively in intact human platelets and lysates with a K_i of 3.7×10^{-4} M. DEHP does not inhibit PLA_2 in whole platelets. Inhibition of PLA_2 by MEHP occurs at only three times the circulating level of MEHP measured in neonates undergoing exchange transfusion and 20-fold the levels experienced by patients during cardiopulmonary bypass. Therefore, infants and adult patients with multisystem failure who accumulate MEHP in their blood may be at risk for decreased platelet function.

Introduction

The commonly used plasticizer in polyvinyl chloride (PVC) plastic, di(2-ethylhexyl)phthalate (DEHP), is a widespread environmental contaminant. Although it is a nongenotoxic carcinogen (*1*) since it does not bind to DNA (*2*), a variety of chronic toxic effects have been shown to occur in rats and mice when DEHP makes up 0.6 to 1.2% of their diet. These include embryotoxicity and teratogenicity (*3*), as well as carcinogenicity (*4*) and testicular atrophy (*5,6*). There is substantial evidence that the toxic effects observed are due not to the parent compound, but to one or more of its metabolites (*7,8*). The initial step in the metabolism of DEHP *in vivo* is its hydrolysis to mono(2-ethylhexyl)phthalate (MEHP). This process also occurs during blood storage in a plastic container (*9*). Thus, red cell concentrates (*9*), platelet concentrates (*9*), albumin (*10*), and cryoprecipitate (*11*) all contain DEHP and MEHP. Although human exposure to MEHP can occur through ingestion (*12*), the most direct and concentrated exposure to MEHP is by transfusion of blood components which have been collected and stored in flexible plastic containers (*9*).

In addition, hemodialysis patients (*13*), hemophiliacs (*11*), and patients undergoing cardiopulmonary bypass (*14*) all have measurable quantities of circulating MEHP, which are cleared within 24 hr if renal function is normal (*14*). The highest levels of MEHP recorded were 15 μg/mL (2.7×10^{-5} M) found in infants undergoing exchange transfusions (*15*).

Although it is difficult to assess the chronic effects of repeated short-term exposure to MEHP, acute effects can be measured. The infusion of MEHP into rats was examined and recently shown by Rock et al. to cause hypotension followed rapidly by cardiac arrest (*16*).

In other studies, Tavares et al. have shown that MEHP inhibits acetylcholine and prostaglandin PGE_2-induced contractions of a rat gastric fundus muscle preparation (*17*). Tavares and Vine also

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there was reduced production of prostaglandins and leukotrienes in rat peritoneal leukocytes in the presence of MEHP and suggested that MEHP may have a similar anti-inflammatory action to aspirin (*18*). However, aspirin inhibits prostaglandin production, but not leukotriene formation, suggesting that MEHP is operating at a level of the liberation of free arachidonic acid (AA) from membrane phospholipids. The arachidonic acid must first be removed from the *Sn*-2 position of the phospholipids by PLA_2 before it can be metabolized to form eicosanoids. In this study, platelets were used to study the inhibitory action of MEHP on PLA_2 in intact suspensions as well as cell lysates. In addition, the type of inhibition and the inhibitor binding constants were determined for the human platelet PLA_2.

Methods

Preparation of Whole Platelets and Platelet Lysates

Blood was collected from randomly selected normal donors who met all criteria for blood donation of the Canadian Red Cross into the standard (citrate-phosphate-dextrose-adenine) CPDA-1 blood bags. The red cells were sedimented at 400*g* for 7 min in a Sorvall RC-3B centrifuge at 22°C (Dupont Co., Newtown, CO). The platelet-rich plasma (PRP) was expressed into the platelet storage bag and then centrifuged at 1300*g* for 6 min at 22°C in a Beckman Model J-6M (Beckman Instruments, Inc., Fullerton, CA). All but 60 mL of the plasma was expressed into the plasma storage bag. The concentrated platelets were left to sit undisturbed at room temperature for 1 hr and then resuspended for 1 hr on a Helmer platelet agitator (Forma Scientific, Marietta, OH) at 6 cycles/min.

Aliquots (1 mL) of platelet concentrate were centrifuged at 1300*g* for 12 min. The supernatant was removed and the platelets were resuspended in 0.5 mL of 0.1 M Tris (trihydroxymethylaminomethane), 2 mM ethyleneglycoltetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA) (TEG) buffer, pH 9.0, which had been warmed to 37°C. A platelet lysate was prepared by overlaying the platelet pellet with 0.5 mL of TEG buffer, pH 9.0, and freezing overnight at −20°C. The platelet count of the suspension prior to freezing or assay was determined using a Coulter Counter Model ZBI (Coulter Electronics Inc., Hialeah, FL).

Phospholipase A_2 Assay

The phospholipase A_2 (PLA_2) assay procedure was a modification of Ballou and Cheung (*19,20*). The assay mixture contained water (15 μL), 68 mM $CaCl_2$ (20 μL), Tris buffer, pH 9.0 (130 μL), and 1-stearoyl-2-[1-^{14}C]arachidonyl L-3-glycerophosphocholine (PC) (Amersham Canada Ltd., Oakville, ON) (10 μL). The solution of PC added to the assay mixture was prepared from a stock solution of PC, specific activity 58 mCi/mM in toluene:ethanol (1:1). The specific activity of the PC was adjusted by mixing the radioactive stock solution with additional PC that was not radioactive, evaporating this mixture to dryness under nitrogen, and redissolving the PC in dimethyl sulfoxide (DMSO). Each assay contained from 0.215 to 2.15 nmole of PC and approximately 40,000 cpm added in 10 μL of DMSO. The reaction was started by the addition of an aliquot of the whole platelet or platelet lysate suspension (25 μL) and incubated at 37°C for various times. The reaction was stopped by the addition of 200 μL of ethanol containing 2% acetic acid and 20 μg arachidonic acid (AA). An aliquot of 200 μL was spotted in a band on a silica gel G thin-layer plate and chromatographed in hexane:ether:acetic acid (70:30:1). The bands corresponding to AA and PC were visualized with iodine vapor, scraped, and counted in 10 mL Ready-Solv-HP scintillation cocktail (Beckman Instruments, Inc., Toronto, Canada), in a liquid scintillation counter (Model LS-7500, Beckman Instruments, Richmond, CA).

The percentage hydrolysis was calculated by the following equation:

$$\% \text{ Hydrolysis} = \frac{\text{arachidonic acid (cpm)} \times 100\%}{\text{phosphatidylcholine (cpm) and arachidonic (cpm)}}$$

$$\text{Rate of hydrolysis (nmole/min)} = \frac{\% \text{ Hydrolysis} \times \text{nmole phosphatidylcholine per assay}}{100 \times \text{incubation time (min)}}$$

The amount of arachidonic acid formed (nmole) per minute was equivalent to the rate of hydrolysis of PC. When the inhibition by MEHP was measured, aliquots of a stock solution of MEHP, 1 mg/mL in methanol, were evaporated to dryness under nitrogen, and the MEHP was redissolved in Tris buffer, pH 9.0, so as to give the required amount of MEHP to be added to the assay mixture.

Results

The kinetics of PLA_2 hydrolysis of phosphatidylcholine (PC) to release arachidonic acid were measured in whole platelets and in platelet lysates (Fig. 1). For whole platelets the hydrolysis was linear up to a maximum of 5% hydrolysis and had a rate of 2 nmole arachidonic acid formed per minute by 10^9 platelets at 37°C. Platelet lysates hydrolyzed the PC 10% under the same conditions producing 3.9 nmole/min/10^9 platelets. Freezing and thawing the platelet suspensions several times did not increase the PLA_2 activity released by freezing overnight. The platelet PLA_2 was saturated at a substrate concentration of 5.4×10^{-3} M for both lysate and whole platelet preparations and was directly proportional to the number of platelets per assay up to 10^8 platelets (data not shown).

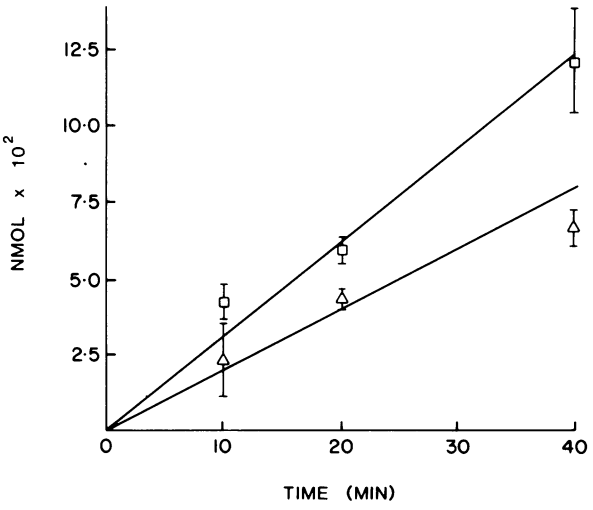

FIGURE 1. Effect of time on the hydrolysis of PC by whole platelet (△) and platelet lysate (□) PLA_2. The assay was performed as described in "Methods," except that incubation times were increased from 10 to 40 min.

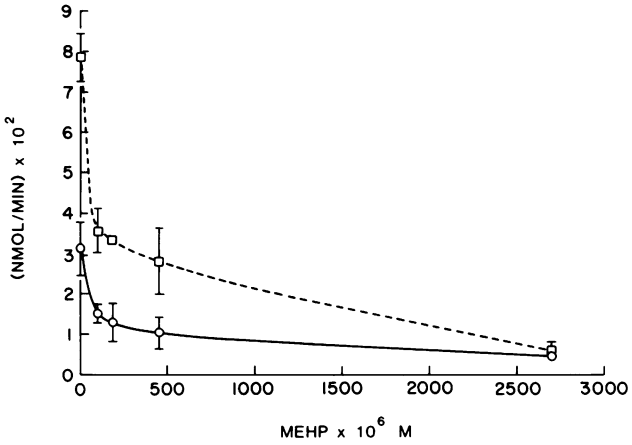

FIGURE 2. Inhibition of whole platelets (O) and platelet lysates (□) by MEHP.

MEHP inhibited the PLA_2 activity of whole platelets as well as platelet lysates (Fig. 2). At $9.0 \pm 0.92 \times 10^{-5}$ M MEHP, the PLA_2 activity in whole platelets was inhibited 50%, whereas 50% inhibition of PLA_2 in platelet lysates occurred at $7.5 \pm 0.07 \times 10^{-5}$ M. Dixon's analysis shows that the MEHP noncompetitively inhibits platelet PLA_2 (*21*) (Fig. 3). The inhibitor binding constant (K_i) for PLA_2 inhibition was the same for the whole platelets and the platelet lysates, 3.7×10^{-4} M.

Discussion

It was possible to measure PLA_2 activity *in vitro* using whole platelets. Previously this assay was used to measure the release of arachidonate from PC using platelet homogenates (lysates) and isolated platelet membrane preparations (*19,20*). The arachidonic acid released from the PC was directly proportional to the number of platelets per assay and followed Michaelis-Menten kinetics. The location of PLA_2 within the platelet membrane in resting platelets has not been defined. It is thought that PLA_2 is on the inner surface of the platelet membrane and that it becomes exposed during platelet activation (*22*). The fact that it was possible to measure hydrolysis of PC using intact resting platelets means that some of the enzyme must have been exposed or some of the platelets were lysed. As there was a twofold increase in PLA_2 activity upon lysis, it is unlikely that small amounts of lysis during platelet preparation gave the PLA_2 activity measured in whole platelets. Also, repeated freezing and thawing up to five cycles gave no further increase in PLA_2. This was confirmed by the fact that the K_i for MEHP was the same for both whole platelets and platelet lysates.

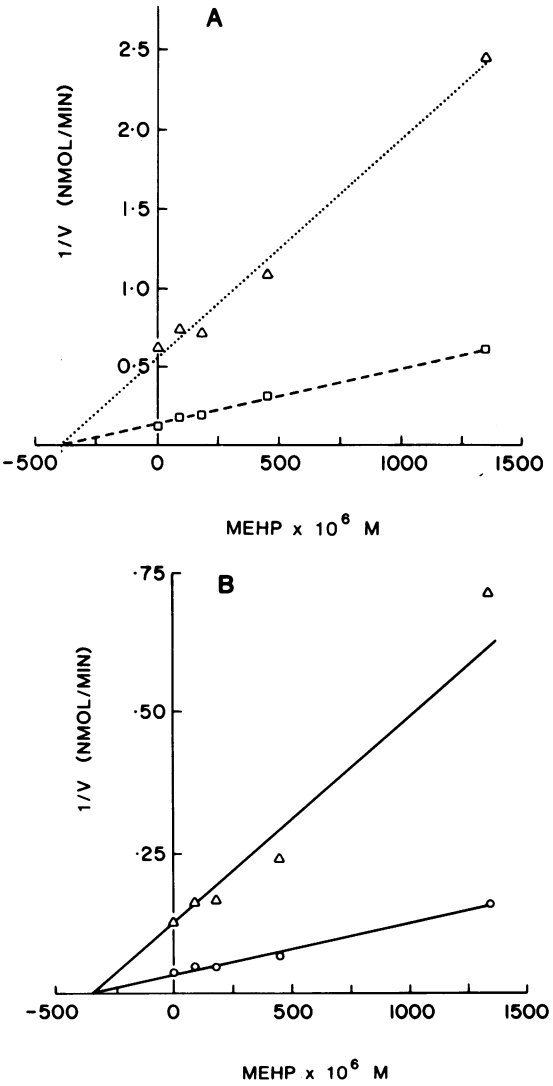

FIGURE 3. (*A*) Determination of the inhibitor constant, K_i, for MEHP measuring whole platelet PLA_2 activity at 10.8×10^{-3}M PC (□) and 2.15×10^{-3} M PC (△). (*B*) Determination of the inhibitor constant, K_i, for MEHP measuring platelet lysate PLA_2 activity at 10.8×10^{-3} M PC (O) and 2.15×10^{-3} M PC (△).

PLA_2 is one of the enzymes involved in arachidonic acid release from membrane phospholipids. Whether or not it is the most important source of arachidonic acid has not been absolutely established for human platelets. It is possible that phospholipase C, formed by diacylglycerol lipase, is responsible for the release of arachidonic acid following platelet activation (*22*). In any case, the fact that it was possible to measure human platelet PLA_2 activity without disrupting the cell made it an excellent system to study the toxic effects of the plasticizer metabolite MEHP in humans.

There is accumulating evidence that it is not DEHP, but its primary metabolite, MEHP, which is responsible for the chronic toxic effects observed when DEHP is fed to rats and mice. This is also true of the acute inhibitory effect on PLA_2. DEHP showed no inhibition of PLA_2 at concentrations 10-fold higher than those of MEHP, but DEHP does not cause hypotension and cardiac arrest in rats. When toxic effects of a compound are observed in animals, a margin of safety (MOS) is chosen based on the severity of the toxic end point and the uncertainty of the data base when extrapolating to human exposure and can vary from 100 (*23*) to 1000 (*24*). The levels that caused the toxic effects we observed in rats corresponded to a MOS of 50 when compared to levels of exposure in a trauma patient during massive transfusion (*25*). This is still 2-fold lower than the lowest acceptable MOS quoted by Lehman and Fitzhugh (*23*) and 20-fold lower than a more recently chosen MOS (*24*).

Since then, we have measured circulating levels of MEHP in clinical situations such as cardiopulmonary bypass and heart transplant patients (*14*) and others have measured circulating levels of MEHP in neonatal exchange transfusions (*15*). Concentrations of 2.7×10^{-5} M have been measured in transfused newborns (*15*) and we have measured 4×10^{-6} M MEHP in the blood of babies at the end of 1 hr of cardiopulmonary bypass (*14*). At the K_i for MEHP, 3.7×10^{-4} M, presumably only 50% of the whole platelet PLA_2 is active. This is only 14-fold higher than the values actually measured in clinical situations. Certainly, platelet PLA_2 plays an active role in the *in vivo* function of human platelets. The rapid response and intricate control mechanisms responsible for the initiation of hemostatic plug formation involves a well-functioning PLA_2. It is very difficult to choose an acceptable MOS when the clinical situation involves compromised patients with multisystem failure.

In rats, DEHP and MEHP have low acute oral toxicity and since these compounds were shown to be nongenotoxic carcinogens (*1*), concern about the chronic toxic effects that occur after long-term exposure by ingestion has been alleviated. Also, elimination of these widespread environmental contaminants from the body occurs rapidly unless renal function is impaired (*14*). However, infants and adult patients with multisystem failure accumulate MEHP in their blood and therefore may be at risk for decreased platelet function.

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