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The Effect of Mono(2-ethylhexyl)phthalate on an Isolated Perfused Rat Heart-Lung Preparation

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Di(2-ethylhexyl)phthalate (DEHP), the plasticizer used in the biomedical production of blood storage bags, hemodialysis systems, cardiopulmonary bypass (CPB) circuitry, and intubation tubes, is extracted from the plastic material when it comes into contact with biological fluids and is converted to its principal metabolite, mono(2-ethylhexyl)phthalate (MEHP). We have shown that MEHP causes cardiac and respiratory arrest, as well as hypotension, when infused into anesthetized rats. Using a well-ventilated *in vitro* rat heart-lung preparation, we investigated the effect of MEHP on pulmonary artery pressure (PAP) and found that MEHP had a hypertensive effect on the pulmonary vasculature ending in constriction and edema. There was a significant increase of 0.58 mm Hg/min in the PAP of isolated rat lungs when perfused with MEHP dissolved in Krebs-Henseleit (K-H) buffer ($p = 0.0003$). The rat lungs that were perfused with K-H buffer only increased 0.094 mm Hg/min during the same perfusion time of 20 min. The water gained during this time was 0.22 g/min with MEHP in the buffer compared to 0.04 g/min with buffer alone. The pO_2 in the effluent did not decrease during the perfusion time. The concentration of MEHP in the rat lungs after perfusion varied from 20 to 40 μg/g. Although the mechanism of action of MEHP on PAP is too complex to be fully elucidated by this model, the increase in PAP which we have demonstrated is significant and adds yet another toxic effect of this major metabolite of the ubiquitous plasticizer, DEHP.

Introduction

Di(2-ethylhexyl)phthalate (DEHP) has been the most commonly used phthalate ester plasticizer in the manufacture of polyvinylchloride-derived plastics, especially in the biomedical production of blood storage bags, hemodialysis systems, cardiopulmonary bypass (CPB) circuitry, and the tubes used for intubation of patients requiring respiratory assistance (*1*). DEHP is extracted from the plastic material when it comes into contact with lipophilic biological fluids, especially blood, and is converted to its principal metabolite, mono(2-ethylhexyl)phthalate (MEHP) (*2*).

It has been known for some time that phthalic acid ester and trimellitic anhydride (TMA) plasticizers have caused a spectrum of respiratory symptoms in workers in the plastics industry. The symptoms for TMA have ranged from asthma-rhinitis syndrome of both delayed and immediate onset (*3,4*) to acute pulmonary hemorrhage and edema (*5*). Ahmad et al. (*6*) reported that seven workers exposed to TMA developed hemolytic anemia and repeated hemoptyses. Earlier, DEHP had been reported to have caused the formation of microemboli, impeding pulmonary blood flow and possibly producing death via the shock lung syndrome that is seen following massive blood transfusions (*7*). This is supported by reports of pulmonary insufficiency and edema in humans following blood transfusions (*8,9*). Autian (*10*) reported that when mice were given ^{14}C-DEHP IV for 7 days, the lungs contained the highest specific activity of any organ studied. Recently, it has been reported that DEHP accumulated in the lungs of neonates requiring intubation for a number of respiratory problems, and it has been suggested that the high levels of DEHP measured may have contributed to the death of these neonates (*1*).

Substantial evidence has shown that the toxic effects observed in animals are due not to DEHP, but to MEHP, and include testicular atrophy (*11*), fetotoxicity/teratogenicity (*12*), and carcinogenicity (*13,14*). In addition, we have shown an inhibitory effect of MEHP, but not of DEHP, on the human platelet enzyme phospholipase A_2 (*15*). MEHP also causes cardiac and respiratory arrest, as well as hypotension, in anesthetized rats (*16*). After showing that both DEHP and MEHP accumulated in the blood of patients undergoing CPB operations for a variety of reasons (*17*), we established that a dose-dependent cardiodepressive effect of MEHP, but not DEHP, occurs in isolated human atrial (*18*) and papillary (*19*) muscle preparations.

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In order to study the respiratory effects of MEHP, we used a well-ventilated *in vitro* rat heart-lung preparation and perfused the lungs with Krebs-Henseleit buffer containing MEHP. The rate of change of pulmonary artery pressure (PAP) with MEHP was compared to a control group perfused with buffer only. The extent of the accumulation of water and MEHP in the lungs during this time was also measured.

Experimental Procedure

In Vitro Rat Heart-Lung Preparation

The rats (Sprague-Dawley, males, 250 g, Charles River, Ontario) were anesthetized with Somnotol (MTC Pharmaceuticals, Canada Packers, Cambridge, Ontario) (70 mg/kg), placed on their backs, and their limbs were taped down. An incision was made cranial from the external genitalia to the tip of the jaw. The strap muscles in the neck area were blunt dissected and the trachea exposed. A cut between two rings of the trachea, just caudal to the cricoid cartilage, was made, and a polyethylene cannula (0.055 inches ID) was inserted and secured with a 0.0 suture. The cannula was connected via silastic tubing to a Harvard Rodent Ventilator, model 683 (Ealing Scientific, St. Laurent, Quebec). The rat was ventilated at a rate of 50/min with a tidal volume of 2.5 cc.

Next, the abdominal wall was cut from the level of the penis to the xiphoid process, and the viscera were placed off center to expose the inferior vena cava. A mixture of 0.45 cc of heparin (1000 IU/mL, equivalent to 100 IU/100 g body weight) and 0.15 cc of a modified Krebs-Henseleit (K-H) buffer (composition is described below) was injected into the inferior vena cava. The diaphragm was cut, the chest opened, the ribs spread apart and the thymus removed. The rat was then exsanguinated by cutting the inferior vena cava. A small incision was made in the right ventricle, and a polyethylene cannula (0.055 inches ID) was inserted into the pulmonary artery (PA). This cannula was secured with a 2.0 suture. The apex of the heart was cut off and the mitral valve pierced to ensure easy outflow of the perfusate.

The lungs and nonfunctioning heart were then excised, and to prevent the entry of any air bubble into the pulmonary vasculature, the cannula was topped off with K-H buffer using a needle. The organs were immediately weighed and then attached to the *in vitro* circuit (Fig. 1). The circuit consisted of a water heater and pump (Techne Tempette Junior TE-8J, Mandel Scientific, Montreal, Quebec) which circulated water from a bath into the external compartment of a heating coil to keep the perfusate at physiological temperature. The perfusate, which was kept in the water bath, was pumped by a Gilson Minipulse 2 peristaltic pump (Mandel Scientific, Montreal, Quebec) into the internal compartment of the heating coil. From there the perfusate entered the PA cannula and the lungs with an initial flow rate of 3.4 mL/ min. Just at the junction of the PA cannula and the heating coil, a T-junction provided for lateral pressure measurement of the PAP via a Statham Gould pressure transducer (Allen Crawford Associates, Ottawa, Ontario). The pressure was then recorded on a Grass 79D oscillograph (Grass Instruments, Quincy, MA). The cannula was initially adjusted so as to read a minimum pressure and from there, the flow rate was modified to one which yielded a stable pressure close to 5 mm Hg. After a period of stabilization of approximately 5 min, the perfusate was changed from the K-H control to K-H containing varying amounts of MEHP. Samples of the perfusate and the effluent were taken at 5-min intervals throughout each experiment for determination of pO_2, using a pH/ blood gas analyzer, model no. 1306, (Fisher Scientific Ltd., Nepean, Ontario) as well as for extraction

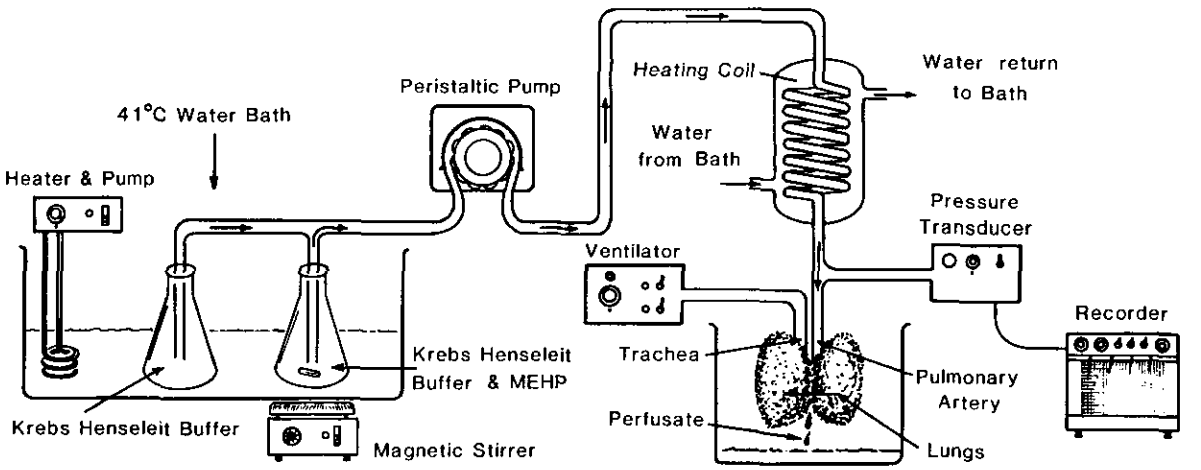

FIGURE 1. The *in vitro* rat heart-lung circuit. Krebs-Henseleit (K-H) buffer and K-H buffer with MEHP (continuously stirred by a magnetic stirrer) is maintained at 37°C in a heated water bath. This water is also pumped through a heating coil to maintain the perfusate at 37°C while it is being pumped by the peristaltic pump at a rate of 3.4 mL/min from the reservoirs to the lungs via the pulmonary artery. The lungs are aerated through the trachea with a mechanical ventilator at a rate of 50/min with a tidal volume of 2.5 cc. The changes in pulmonary artery pressure are monitored by a pressure transducer and recorded. The details of the surgical procedure for the preparation of the lungs are described in "Experimental Procedure."

of MEHP for analysis by HPLC in order to determine the exact concentration. At the end of the experiment, the lungs were reweighed to permit the calculation of the water gained and then frozen for later MEHP extraction.

Preparation of the Perfusate

The modified K-H buffer was prepared with the following composition (millimolar): NaCl, 109; KCl, 4.7; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; glucose, 11; fumaric acid, 5; glutamic acid, 5; sodium lactate, 5; zinc insulin 25 mU/mL; and amino acids and vitamins as in Eagles medium (*20*). Human serum albumin obtained from the Canadian Red Cross was added to give a final concentration of 5%. The final pH was adjusted to 7.4 with sodium hydroxide (10N).

MEHP (Fairfield Chemical Co., Inc., Blythewood, SC) was dissolved in 0.15 M $NaHCO_3$ at a concentration of 10 mg/mL and added to the K-H buffer in amounts varying from 15 to 150 μg/mL just before the perfusion was started.

Analysis of Rat Lungs and Perfusates for MEHP

Specimens of 100 mg from the rat lung preparation were extracted for accumulation of MEHP. Extraction of the tissues was done according to the procedure of Acott et al. (*21*), using a ground glass homogenizer and sodium sulfate to break up the tissue. HPLC, model no. 5000 (Varian Associates, Georgetown, Ontario) was used to determine the concentration of MEHP (*22*) in the extracted lungs as well as in aliquots of the perfusates and effluents.

Electron Microscopy of Perfused Rat Lungs

The lungs were inflated with and immersed in 2.5% glutaraldehyde. A biopsy of the upper segment of each of the lower lobes of each of the rats was taken after fixation. They were examined morphologically, with the observer blinded as to whether individual specimens were MEHP exposed or controls. The tissue was then washed in cacodylate buffer and osmicated in 1% osmium tetroxide in 0.1 M sodium cacodylate with 1% potassium ferricyanide. The tissue was en bloc stained with saturated aqueous uranyl acetate and blocked in Epon-Araldite. The blocks were sectioned with a glass blade and thick 1-μm sections prepared, stained with methylene blue, and examined by light microscopy. Thin 5 to 600 Å sections were prepared with an ultramicrotome and diamond knife, stained with lead citrate, and examined on a Philips 301 electron microscope.

Results

When the lungs were perfused with K-H buffer alone, there was a very small change in the increase in PAP during the perfusion time. The maximum time of perfusion was close to 20 min, after which time the edema became too great and the perfusion rate decreased with a very sharp increase in PAP. During the 20-min period, the rate of water gain was < 0.1 g/min, which was acceptable for this type of preparation (*23*) (Table 1).

Table 1. Effect of perfusion of buffer and MEHP on rat lungs.

MEHP, μg/mL	Time with MEHP, min	Total time perfused, min	Rate of pressure change, mm Hg/min	Weight gain, g/min
0	0	31.00	0.153	0.022
0	0	21.00	0.024	0.046
0	0	27.75	0.180	0.017
0	0	14.50	0.035	0.020
0	0	15.00	0.017	0.075
0	0	19.00	0.092	0.045
0	0	20.00	0.088	0.066
0	0	21.75	0.161	0.031
Mean		21.25 (2.02)[a]	0.094 (0.023)	0.04 (0.008)
15.6	12.50	18.00	0.68	0.213
16.6	7.50	14.00	0.800	0.192
25.7	17.50	21.75	0.114	0.229
31.82	18.50	25.00	0.135	0.003
32.2	10.50	16.00	1.095	0.228
34.8	22.25	27.75	0.225	0.350
39.3	14.00	21.25	0.286	0.100
44.48	10.75	16.25	0.837	0.317
48.7	14.00	18.00	0.143	0.517
56.8	10.50	18.75	0.500	0.167
58.0	7.75	13.50	1.806	0.426
59.8	8.00	13.50	0.688	0.312
60.77	16.50	21.50	0.652	0.173
61.6	12.50	18.25	1.000	0.333
66.23	24.00	28.00	0.208	0.125
68.25	23.25	29.75	0.366	0.121
87.43	13.50	20.00	0.815	0.174
90.29	15.25	22.50	0.705	0.217
91.76	15.50	20.75	0.726	0.197
107.3	27.25	33.75	0.303	0.158
112.2	17.75	23.00	0.648	0.275
120	13.00	18.00	0.308	0.069
150	22.00	27.00	0.324	0.120
Mean		21.5 (1.12)	0.58 (0.08)	0.22 (0.02)

[a] Standard error of the mean in parentheses.

The range of values obtained with controls for both increase in PAP and water gain are presented in Table 1. When MEHP was dissolved in the K-H buffer and perfused through the lung preparation, there was a rise in the rate of increase of PAP (mm Hg/min) during the 20-min perfusion period along with a greater increase in water gain (Table 1). The values for MEHP concentration in the perfusate ranged from 15 to 150 μg/mL. Both the increase in PAP and water gain showed a poor correlation overall with the increase in the concentration of MEHP or time of perfusion with MEHP. There was considerable variability in the response of each animal ($r = 0.55$ and $p = 0.0023$ for MEHP and PAP). However, when the group of experimental lungs were analyzed as a whole, there was a significant effect on the PAP ($p = 0.0003$) if MEHP was present in the K-H perfusate when compared with the controls when only K-H buffer was perfused (rank sum test of presence of MEHP versus rate). The rate of increase was 0.58 ± 0.08 mm Hg/min with MEHP and only 0.094 ± 0.023 mm Hg/min with buffer alone (Table 1). There was also a large difference (5-fold) in the amount of edema in the experimental lungs, 0.22 ± 0.02 g/min increase in weight (Table 1) when compared to the

Table 2. Gas analyses of rat-lung perfusate.

Group	pO_2, mm Hg Start	End
Control	180	141
Control	134	121
Experimental	152	145
Experimental	139	135
Experimental	139	117
Experimental	139	114

controls when the rate of water accumulation was only 0.04 ± 0.008 g/min during the perfusion period.

Both the experimental and control lung preparations were well aerated during the perfusion period since the pO_2 measured in the effluent did not change significantly from the beginning to the end of the perfusion time (Table 2). When the rat lungs were extracted and analyzed for MEHP, there was an accumulation of MEHP in the tissue that varied from 20 to 40 μg/g. However, this was always lower than the concentration of MEHP in the perfusate. The histopathologic examination of the lung preparations showed no discernible differences at the light or ultrastructural levels when the MEHP-exposed and control tissues were compared.

Discussion

We had observed that MEHP caused cardiac and respiratory arrest when infused into anesthetized rats when we were monitoring heart rate and blood pressure, both of which were depressed (*16*). In an intact, anesthetized animal, many factors can contribute to the pathogenesis of the cardiodepressive effect. We had already shown the cardiodepressive effects in isolated human atrial trabecular muscle preparations (*18,19*). Therefore, in order to isolate the effects of MEHP to the respiratory system, we developed an *in vitro* rat heart-lung preparation where the heart is nonfunctional and the lungs are well aerated mechanically. Our results have shown a significant effect on PAP when MEHP is present in K-H perfusate ($p = 0.0003$).

The lack of a good correlation ($r = 0.55$) between increasing concentrations of MEHP and PAP and water gain and the high variability in the response from animal to animal was probably due to MEHP having more than one effect on the lungs as well as the direct effect of perfusion on water gain in this preparation which is not related to MEHP (*23*). The effects of MEHP on the increase of PAP and water gain are related, but probably are two different phenomena which have different mechanisms. This may be part of the reason for the lack of good correlation between MEHP concentration and the increase in PAP. The biological variability in the two responses of the pulmonary artery to MEHP, i.e., water gain and increased pressure, may be more than additive. Certainly, water gain alone will also cause an increase in PAP (*23,24*). In addition, the variation in the perfusion times with and without MEHP make correlation to one variable very difficult. Perhaps the lack of a dose response indicates that there are different thresholds for an all-or-none phenomenon for both the hypertensive and water gain effects.

There are several possible mechanisms by which MEHP may exert its effects on PAP and water gain in this preparation. When considering the 6-fold increase in the PAP, it is important to note that the lung is affected by changes in hydrostatic and oncotic pressures as well as ventilation or ischemia. All of these factors are determined by interactions of the pulmonary cellular components of the vasculature and the fluid flowing past them. Although the smooth muscle cells are important in maintaining the tonicity of the vessel wall, it is the endothelial cell that is important in controlling PAP by altering the vasoactivity by the following mechanisms: *a*) control of release of vasoactive substances that influence vascular tone at the site of or at a site distal to that of its formation/alteration/uptake (*24*); *b*) regulation of smooth muscle tonicity through the formation of a mediator; and *c*) control of viscosity of the fluid by interacting at the endothelial cell surface (*25*).

For example, vasodilatory substances to the endothelium, such as acetylcholine, bradykinin, and serotonin can become vasoconstrictive when introduced to denuded pulmonary vessels (*26*). Ischemia/hypoxia also causes a hypotensive factor to be released from endothelial cell walls (endothelium-derived relaxing factor [EDRF]) (*27*). MEHP may be exerting its action by interfering with one or more of the three mechanisms described above, by damaging the endothelium, which then reverses the relaxation effects of EDRF to a contraction (*26*). However, there have been several studies that suggest that MEHP acts through the cholinergic system. These include the inhibition of acetylcholine-induced rat gastric muscle contraction (*28*), as well as our own studies which demonstrated that the cardiodepressive effect of MEHP in the atrial human *in vitro* muscle preparation could be blocked by atropine (*19*). Pfuderer et al. (*29*) showed that atropine also blocked the heart depressor effects of several phthalate esters in the goldfish. Since the rat lung preparation described in this study is well-ventilated, the increase in PAP caused by MEHP is consistent with a cholinergic effect, as acetylcholine can be vasoconstrictive if pulmonary vessels are damaged and if there is no hypoxia (*26,27*).

The 5-fold increase in water gained during the perfusion period indicated that there was an alteration in the pulmonary vasculature caused by MEHP, although no morphological changes were apparent other than edema, which was evident in both the control and MEHP-perfused lungs. It appears that the permeability of the pulmonary vasculature may have been altered without visible ultrastructural changes occurring.

Edema is caused by changes in the capillary and tissue hydrostatic and oncotic pressures regulating the transcapillary gradient as described by the Starling hypothesis. Any minimal change in either the hydrostatic pressure (such as an increase) within the capillary or a decrease in its oncotic pressure will lead to increased fluid leakage out of the capillary into the interstitium, resulting in edema. In this preparation, maximal effort was placed in maintaining a physiological intracapillary hydrostatic as well as oncotic pressure. However, it is difficult to control interstitial and intracellular

hydrostatic and oncotic pressures in these *ex vivo* preparations. Others (*23,24*) have had similar problems. Nonetheless, it is apparent that more than just the *in vitro* artifactual edema was present in the experimental lungs since there was a significant increase in the water gained per minute when MEHP was present. It is possible that the edema observed was the result of an effect of MEHP on the endothelial cell and its functions, since other biologically active substances such as prostaglandins can do the same by increasing the endothelial cell junctional gaps, allowing water to leak out (*30*). Although this is only a hypothetical explanation, it should be explored further.

It is evident, however, that the mechanism of action of MEHP on the pulmonary artery is too complex to be fully elucidated by this model. Nonetheless, the increase in PAP and edema that we have demonstrated by using MEHP is significant and adds yet another toxic effect of this major metabolite of the ubiquitous plasticizer, DEHP.

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