Effect of glycerol-induced acute renal failure and di-2-ethylhexyl phthalate on the enzymes involved in biotransformation of xenobiotixs

Etelka **Kertai**[1], G. **Hollósi**[2], Judit **Kovács**[3], V. **Varga**[2,4]

[1] Department of Pharmaceutical Technology,
[2] Department of Animal Anatomy and Physiology,
[3] Institute of Pathology University of Debrecen, Debrecen, Hungary
[4] Brain Research Center, University of Tampere Medical School, Tampere, Finland

The effects of di-(2-ethylhexyl)-phthalate (DEPH) on the levels of cytochrome P-450 and b_5 monooxygenases were studied in the rat kidney and liver in acute renal failure induced by glycerol. Intramuscular injection of glycerol ($50\%, 10 \text{ ml·kg}^{-1}$) to rats produced proximal tubular damage and acute renal failure. The indicators of renal function, serum urea and creatinine significantly increased (480 and 350 percent, respectively). In control and glycerol-treated animals DEPH had no significant effect on the concentrations of serum urea and creatinine. Twenty-four hours after glycerol injection the total amount of cytochrome P-450 and b_5 significantly decreased in renal but increased in liver microsomal fractions. Moreover, 48 and 72 hours after glycerol injection the level of cytochrome P-450 and b_5 significantly increased in both organs. A single dose of DEPH (2 ml·kg^{-1}, i.p.) also elevated the total cytochrome P-450 and b_5 in control animals. This enhancing effect of DEPH was additive to that of glycerol in glycerol-induced acute renal failure.

These results indicate that DEPH and glycerol evoked pathological changes may affect the metabolism of xenobiotics plus endogenous hormones in the liver and in kidney.

Keywords: glycerol induced acute renal failure, rhabdomyolysis, myoglobinuria di-2-ethylhexyl phthalate, cytochrome P-450, cytochrome b_5, xenobiotics, acute renal failure, glycerol, proximal tubular damage, metabolism of xenobiotics

Correspondence should be addressed to
Ass. Prof. Gábor Hollósi
Debrecen University
Department of Animal Anatomy and Physiology
H-4010 Debrecen, P.O. Box 15, Hungary
Phone: 36-(52)-512-900 ext. 2739
E-mail: hollosig@delfin.klte.hu

Medical devices such as transfusion sets, dialysis units, disposable syringes, vascular grafting materials, catheters and storage bags for blood, dialysis and infusion solutions are often made of plastic. These plastic materials are composed of polyvinyl chloride (PVC) and plasticizers such as di-(2-ethylhexyl)-phthalate (DEPH). In some plastic blood packs the amount of DEPH is 35–40% of the plastic materials [3]. Since DEPH is not covalently bound to PVC, it may be liberated into fluids and accumulate as xenobiotic in blood and organs of patients. Patients suffering from acute and chronic renal failure (ARF and CRF) are often subjected to hemo- and peritoneal dialyses and receive intravenous infusions. For these patients DEPH is a potentially hazardous compound. In rodents, long-term administration of DEPH and dibutyryl phthalate causes renal cysts [39] and proliferation of peroxisomes in the liver and kidney. Phthalate esthers increase the liver mass and the concentration of cytochrome P-450 enzyme system [21].

The cytochrome P-450 (CYP superfamily) of mixed function oxidases (MFO) are the principal enzymes in the metabolism of xenobiotics. They play a role in the metabolism of fatty acids and steroids, biotransformation of toxins, oxidation of nitrogen, alcohol and aldehyde, oxidative deamination, reduction of aldehydes and ketones and hydrolysis and demethylation reactions. They are heme-containing enzymes that serve as the terminal oxidase component in the electron transport system of the endoplasmatic reticulum. In general, the second component of the system is a flavoprotein cytochrome P-450 reductase that transfers reducing equivalents from NADPH to cytochrome P-450. In addition, cytochrome b_5 and cytochrome b_5 reductase can also donate an electron from NADH to cytochrome P-450 [18]. The concentrations of these enzymes are highest in the liver [10]. In the kidney, the highest concentrations are found in the cortex and proximal tubular epithelium [8, 18, 42].

Recently, important insights into the pathophysiology of different types of ARF have emerged [41], but only a few studies were dealing with the metabolism of xenobiotics during renal failure [5, 31]. We investigated here the effects of ARF and DEHP on the total amounts of cytochrome P-450 and b_5 enzyme proteins in the rat kidneys and liver. Acute renal failure was produced by the intramuscular injection of glycerol as an experimental model of rhabdomyolysis and myoglubinuria [34, 35].

Materials and Methods

Chemicals

Di-2-ethylhexyl phthalate (>97% pure) was purchased from Fluka AG (Buchs, Switzerland). All other reagents were obtained from Sigma (USA) or Boehringer Manheim (Germany).

Experimental model of acute renal failure

Adult, male albino rats of Wistar strain (239±20 g) were housed six per cage and divided into four experimental groups: controls (A), and glycerol-treated (B), DEHP-treated (C) and glycerol+DEHP-treated (D) rats. The rats were deprived of drinking water for 24 h but had free access to food. Acute renal failure was produced by intramuscular injection of glycerol as in [5]. An intramuscular injection of 50% v/v glycerol in sterile saline (0.9% NaCl solution) was given at a dose of 10 ml·kg^{-1}. Control animals were injected with sterile saline (10 ml·kg^{-1}). Drinking water was immediately avaible for rats after the injections. Twenty-four hours after the injections of glycerol or saline, the rats in groups C and D were injected intraperitonally with a dose of 2 ml·kg^{-1} DEHP. The injections were always given between 2 and 3 p.m.

Biochemical investigations

Serum creatinine and urea were measured by the standard spectrophotometric methods [12]. The *microsomal fractions* of kidney and liver were prepared by the method of Miles et al. [22]. Briefly, the kidney cortex and liver were homogenized (0.25 g tissue/ml) in ice-cold buffer with a motor-driven all-glass Potter-Elvehjem homogenizer. Homogenising buffer A was composed of 0.05 M Tris-HCl, 0.154 M KCl, 0.2 mM EDTA and 1.0 mM dithiothreitol. The homogenate was first centrifuged for 20 min at 9,000 g. The pellet was discarded and the supernatant recentrifuged at 105,000 g for 60 min. The pellet was resuspended in buffer B (100 mM Tris-HCl, pH 7.4, 0.2 mM EDTA and 1 mM dithiothreitol) and the centrifugation was repeated at the same centrifugal force. The pellet was finally suspended in buffer B (10 mg protein/ml) and used as microsomal fraction. The whole procedure was done at 4 °C. *Protein content* was measured by the method of Lowry et al. [19] using bovine serum albumin as standard. The *amounts of total microsomal cytochrome P450 and cytochrome b_5* were estimated according to the method of Omura and Sato [27].

Morphological studies

Kidney and liver slices were fixed in phosphate-buffered 10% formalin. Five μm thick paraffin sections were cut and subsequently stained with hematoxylin and eosin and examined by a light microscope.

Ultrastructural studies

The kidney and liver samples were processed for electron microscopy using the method of Okuda et al. [25]. The specimens were fixed in 1.4% glutaraldehyde buffered with 0.1 M cacodylate, postfixed in ice-cold 1% osmium tetroxide in the same buffer for 2 h, dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with JEOL 200 type of electron microscope.

Statistical analysis

The results are given as mean values, ±S.D. Statistical analysis were done by the non-paired Student's-test [32]. The probability level (p) of <0.05 was considered statistically significant.

Results

The intramuscular injection of 50% glycerol produced an acute renal failure leading within 24 h to the uremic state. The concentrations of urea increased 4.3- and 4.8-fold and creatinine 3.2- and 3.5-fold after 24 and 48 h, respectively (Figs 1 and 2).

The glycerol injection alone caused massive tubular epithelial cell swelling, vacuolar degeneration, necrosis, desquamation of cells and cast formation. It was accompanied by glomerular dilatation (Fig. 3B) and increased diameters of capillaries (Fig. 3C). Electron microscopic examinations revealed characteristic alterations in the tubule cells. The mitochondria were enlarged in some parts with distended cristae. In addition, the destruction and lysis of the cristae resulting in a vacuolated pattern and inclusion bodies surrounded by membranes were also discernible within the mitochondria (Fig. 3D). Two and three days after the glycerol injection number of cells were dividing in proximal and distal tubules, indicating regeneration of kidney tissue.
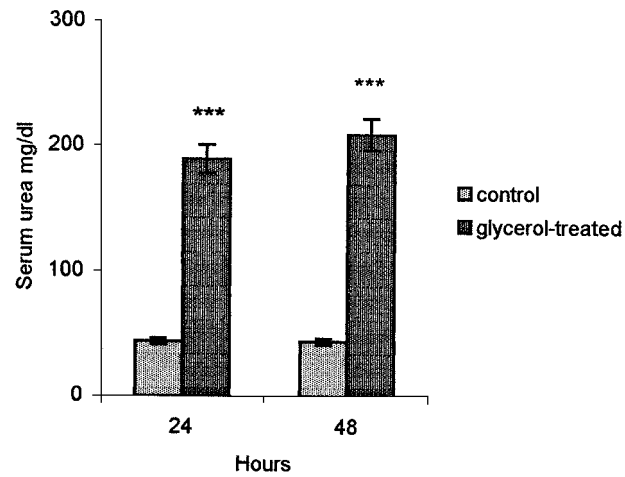
Fig. 1. Serum urea level in glycerol-treated rats. Mean values ±S.D., N = 5, ***p<0.001, when comparing glycerol-treated and control rats
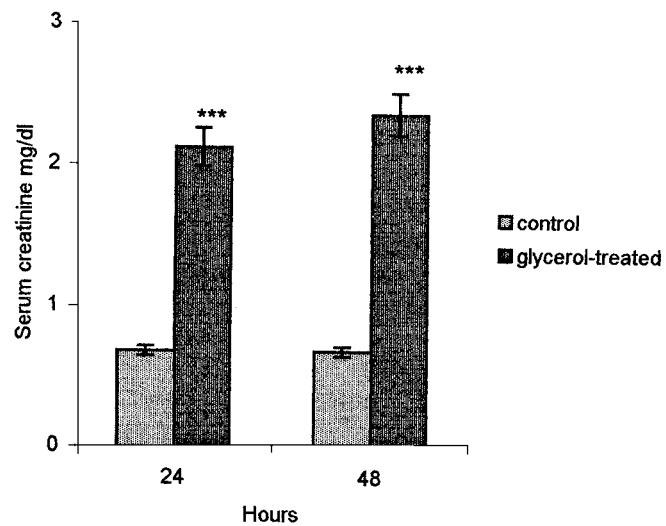

Fig. 2. Serum creatinine level in glycerol-treated rats. Mean values ±S.D., N = 5, ***p<0.001, when comparing glycerol-treated and control rats
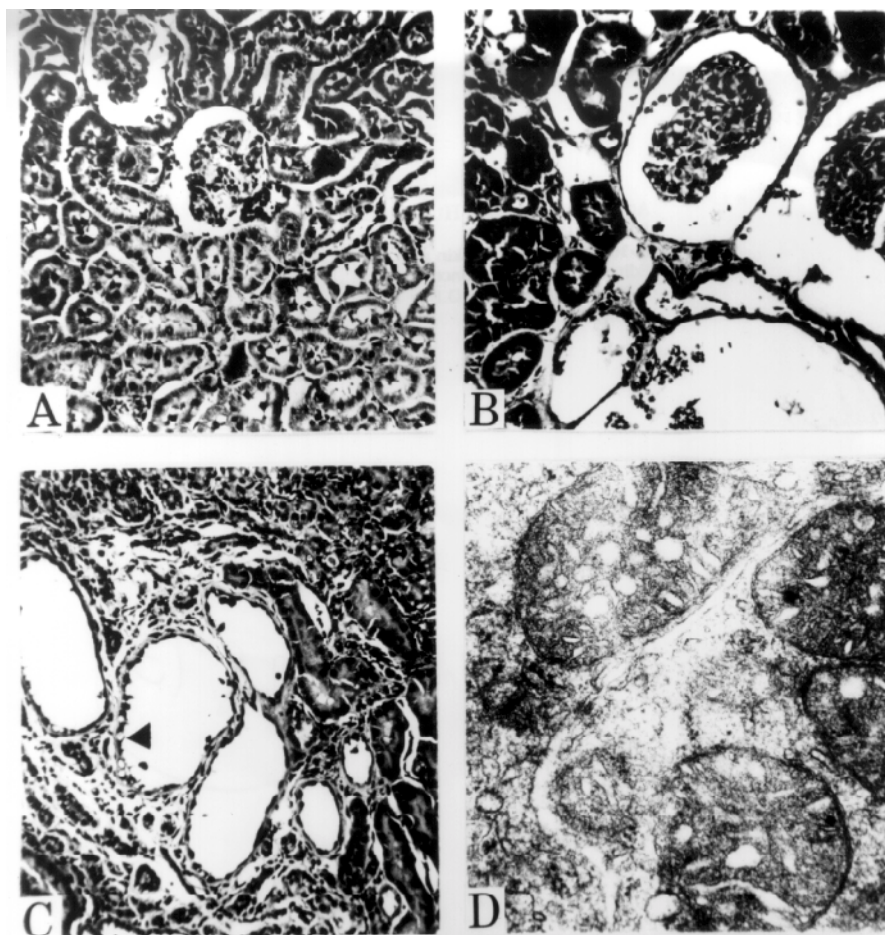
Fig. 3. Kidney sections from control and DEPH-treated rats in glycerol-induced acute renal failure. A–C. Light microscopic photomicrographs (magnification: ×200). A-section of an intact kidney with the normal size of corpusculum renis and nephron. B-section of kidney from DEPH-treated rat suffering from acute renal impairment, a glomerular dilatation can be seen. C-section the same as B with vacuolization of tubular and capillar endothel cells. D-section, fine structure of mitochondria from glycerol and DEPH-treated animals. (magnification: ×30,000). Enlarged mitochondria with slight distention, destruction and lysis of cristae

In all livers examined the wast majority of cells appeared normal. There was no evidence of serious hepatocellular damage in the rats with glycerol-induced ARF. No significant morphological changes were visible in the liver of the glycerol+DEPH-treated rats, but in some cells hydropic vacuolation was evident upon light microscopic examinations (not shown).

Table I

Changes in the absolute and relative kidney mass of glycerol and DEHP-treated animals

Experimental groups	Kidney weight (g)	Increase (%)	Relative kidney weight (g/100 g body w.)	Increase (%)
Controls	1.36 ± 0.15		0.54 ± 0.10	–
Glycerol treated	1.95 ± 0.05**	43.31	0.71 ± 0.02**	30.50
DEHP treated	1.50 ± 0.11*	10.29	0.61 ± 0.09*	13.32
Glycerol+DEHP treated	2.43 ± 0.20***	80.33	0.89 ± 0.02***	63.97

*P<0.01, **P<0.05, ***P<0.001

Results are the mean values (±S.D) of five animals.

The kidney mass and relative mass of kidney significantly increased after glycerol and DEHP injections (Table I). DEPH caused further tissue swelling in the kidney and a number of vacuola was discernible in capillaries (see Fig. 3C and arrow) applied after glycerol DEHP increased the mass of kidney by 78 percent, indicating swelling of tissue.

In glycerol-induced renal insufficiency, the total amounts of cytochrome P-450 and b_5 significantly decreased in the kidney by 21±4 and 18±3%, but increased in the liver microsomal fraction by 15±3 and 17±2%, respectively, after 24 hours (Figs 4, 5, 6 and 7).
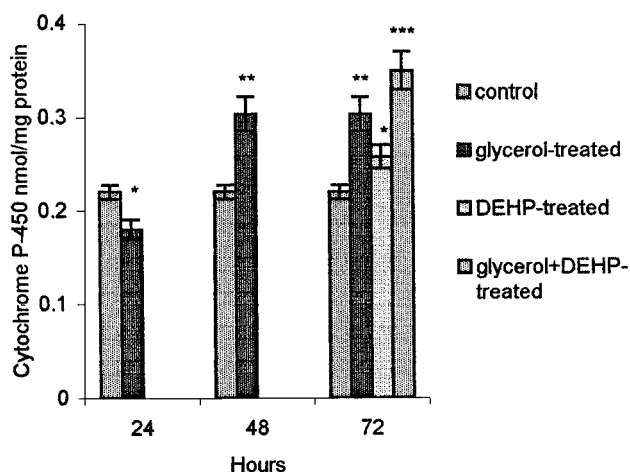

Fig. 4. Effect of glycerol and DEHP on the level of microsomal cytochrome P-450 in the kidney. Mean values ±S.D., N = 5. *p<0.05, **p<0.01, ***p<0.001, when comparing DEHP-, glycerol-, Glycerol and DEHP-treated and control rats
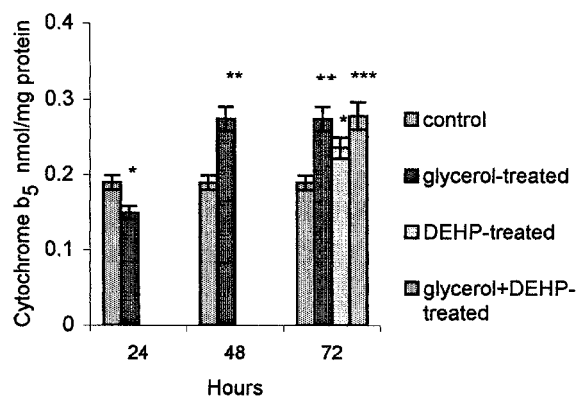
Fig. 5. Effect of glycerol and DEHP on the level of microsomal cytochrome b5 in the kidney. Mean values ± S.D., N = 5, *p<0.05, **p<0.01, ***p<0.001, when comparing DEHP-, glycerol-, Glycerol and DEHP-treated and control rats
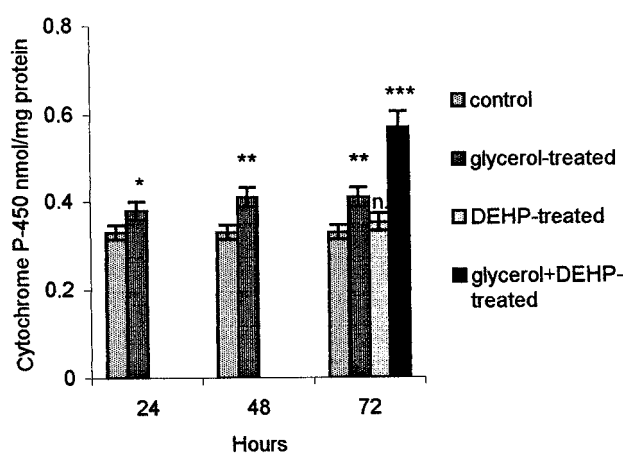

Fig. 6. Effect of glycerol and DEHP on the level of microsomal cytochrome P-450 in the liver. Mean values ± S.D., N = 5, *p<0.05, **p<0.01, ***p<0.001, when comparing DEHP-, glycerol-, Glycerol and DEHP-treated and control rats

Two and three days after the glycerol treatment a significant increase in the concentration of enzymes was detected in the microsomal fractions of both organs. A single dose of DEHP also elevated the level of these enzymes, but the inductive effect of glycerol was greater than that of DEHP. This latter affect was additive to the effect of glycerol (Figs 4, 5, 6 and 7).
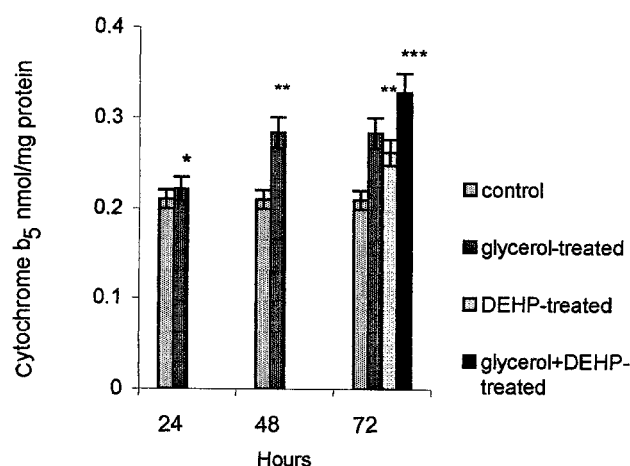
Fig. 7. Effect of glycerol and DEHP on the level of microsomal cytochrome b_5 in the liver. Mean values ±S.D., N = 5, *P<0.05, **P<0.01, ***P<0.001, when comparing DEHP-, glycerol-, Glycerol and DEHP-treated and control rats

Discussion

Crush syndromes, ischemia, metabolic disorders, inflammatory and intrinsic myopathies, excessive physical exercise, alcoholism, drugs, toxins, hypophosphatemia, hypokalemia often lead to degeneration of striated muscle. Rhabdomyolysis may result in myoglobinemia, myoglubinuria and finally in acute tubular and renal failure. Approximately 15% of acute renal failures are caused by myoglobin- and hemoglobinuria [41]. The most widely used animal model of acute renal failure is produced by intramuscular injection of hypertonic glycerol [14]. This intervention consistently produces severe acute tubular necrosis which is reminiscent in its clinical course of crush syndrome [34, 35]. In our experiments, morphological and functional changes were similar to those previously published [4, 35]. The concentrations of urea and creatine increased by 3 to 5 times in serum, indicating a rapid decrease in renal function after glycerol injection (Figs 1 and 2). The morphological changes at 24 h point to epithelial cell degeneration, mainly in the proximal tubules. This may explain the significant decrease in the concentrations of cytochrome P-450 in the kidney microsomal fraction. On the second and third day, an increase in the amounts of cytochrome P-450 and b_5 enzymes were found in the kidney and liver. The former finding can be explained by regeneration of the epithelial cell layer in the proximal tubules. These findings differ from those encounterd in chronic renal failure [15].

The surgically evoked renal insufficiency does not affect the levels of cytochrome P-450 and b_5 in the remnant kidney [15].

The mechanism by which glycerol causes enzyme induction is likely to be multifactorial, similarly to the pathogenesis of glycerol-induced ARF [34]. Glycerol causes muscle injury, myolysis, hemolysis and exposes the kidney and liver to a large burden of heme proteins, myoglobin and hemoglobin. In addition, it diminishes blood volume, which is associated with renal vasoconstriction. Hypovolemia (dehydration) and vasoconstriction are the critical factors which predispose to myoglobinuric acute renal failure. The decrease in renal blood flow diminishes the glomerular filtration rate, resulting in hypoperfusion and depletion of ATP in tubular cells [6, 34, 41]. Furthermore, the readily filtered myoglobin may precipitate (cast formation) and stop flow of urine, which may facilitate the uptake of heme proteins to tubule cells [41]. The porphyrin rings of heme are degraded by heme oxygenase (HO), a member of the cytochrome P-450 superfamily. It is the rate-limiting enzyme in heme catabolism and oxidatively degrades heme into biliverdin, iron and carbon monoxide [20]. In the kidney, the liberation of iron from cytochrome P-450 enzymes has also been demonstrated by Baliga et al. [4]. This may explain the decrease in the level of P-450 enzymes in the kidney during the first day (Figs 4 and 5). Myoglobinemia probably facilitates the uptake of heme-proteins to liver cells. It may thus induce HO that increases the concentration of P-450 enzymes in the liver microsomal fraction (Figs 6 and 7). In the kidney, the induction of HO by myoglobin has been demonstrated by Nath et al. [23]. The localization of inducible HO (HO-1) protein is restricted to tubule cells as a response to $HgCl_2$ induced acute renal failure [13]. The HO-1 is considered to be a stress protein [36]. In the present study HOs may therefore paradoxically contribute to the increase in the concentration of P-450 in the liver already 24 h after the glycerol injection and later in the regenerating renal cells.

On the other hand, glycerol as a substrate for the enzymes CYP2E1 subfamily (a member of cytochrome P-450 superfamily) may prolong the half-life of this enzyme. Addition of glycerol to the culture medium of HEPG2 cell line has enhanced the activity, amount and half-life of CYP2E1 [40]. Glycerol in protein-bound form may inhibit degradation of CYP2E1 causing a relative increase of this enzyme in both organs.

It is of interest that DEHP caused a significant elevation in the amounts of cytochrome P-450 and b_5 in the rats injected only once with DEHP without glycerol. Such a change has not occurred when the renal failure has been induced by a surgical intervention and no significant changes were discernible during the first week. However, after two and three weeks, cytochrome P-450 significantly also increased in CRF [15]. DEPH induces proliferation of mitochondria and peroxisoma and modifies the peroxisomal enzymatic pattern (catalase, D-amino acid oxidase, palmitoyl-CoA

oxidase, arachinoidic acid hydroxilase, etc.) in the liver, kidney and brain [1, 7, 11, 24, 28, 33]. In the kidney, a DEHP-dependent CYP4A enzyme synthesis and acyl-CoA oxidase are also induced [24]. However, not all the DEHP-induced changes of peroxisomal emzyme activities are directly related to peroxisoma proliferation [28].

Two groups of patients are particularly prone to be exposed to high concentrations of plasticizers, namely those on regular hemo- and peritoneal dialysis and patients requiring repeated transfusions of whole blood and platelets [9, 30]. DEPH exposure can be as high as 150 mg per dialysis [9]. In experimental animals, high levels of DEPH have been found in the liver, heart, lung, kidney and fat during the first hours after administration of DEPH [26]. DEPH induces hepatocellular neoplasms in rats and mice [17], liver lesions in rhesus monkeys [16] and enhances cell replication in the kidney [38]. This compound is rapidly eliminated by kidney and liver [37] being metabolized to mono-(ethylhexyl)-phthalate (MEPH), 2-ethyl hexanoic acid and phthalic acid. In cultured kidney epithelial cells in *in vitro* MEPH exerts marked toxic effects. It dose-dependently decreases cell viability, alters organization of F-actin (stress fibers) and leads to lysis of cells [29]. DEPH and its metabolite MEPH may thus cause further damage in kidney especially in ARF and CRF.

ARF changes the pharmacokinetics and excretion of cyclosporine and indocianine green by liver [5, 31]. The kidney also actively metabolizes many hormones and xenobiotics [2]. Our results may indicate that hepatic and renal biotransformation of exo- and endogenous compounds may be affected by DEPH in ARF.

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