Short-term effects of chlorophenols on the function and viability of primary cultured rat hepatocytes

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Abstract. Primary cultured rat hepatocytes were used as an experimental model to detect adverse effects of five chlorophenols (CP) in vitro (penta-CP, 2,3,4,5-tetra-CP, 2,4,5-tri-CP, 2,4-di-CP, and 4-mono-CP). Monolayer cultures were exposed to the test compounds for 1 h, and concentration-response curves were established with respect to the effects on phase I and phase II metabolism of 7-ethoxycoumarin (7-EC) and on cellular ATP content. All CP tested inhibited the O-dealkylation of 7-EC, with half-maximum effective concentrations (EC$_{50}$) ranging from about 36 μM for the three highest chlorinated phenols to 215 μM for 4-mono-CP, which proved to be least effective. The subsequent conjugation of the primary metabolite 7-hydroxycoumarin was even more sensitive towards CP exposure than the O-deethylation process. The concentrations which reduced the percentage of conjugated metabolite to 50% of the respective control cultures ranged from 7 μM for penta-CP to 48 μM for 4-mono-CP. Treatment of cultured hepatocytes with CP additionally resulted in a depletion of cellular ATP at EC$_{50}$ concentrations ranging from 6 μM for penta-CP to 1330 μM for 4-mono-CP. Cellular viability, as measured by the leakage of lactate dehydrogenase from the cells, was not affected by any of the CP within the 1-h exposure period.

Key words: Cultured hepatocytes – Chlorophenols – 7-Ethoxycoumarin – Phase I and phase II metabolism – ATP

Introduction
Within the group of industrial chemicals, chlorinated phenols are of great significance because of their world-wide application as fungicides, bactericides and herbicides. Various studies in vivo and in isolated subcellular systems like mitochondria, microsomes and postnuclear fractions have demonstrated their toxicity (for review see Ahlborg and Thunberg 1980). In the course of the development of more complex in vitro systems for the toxicity screening of chemicals, different cellular models have been applied to evaluate the toxicity of chlorophenols (CP) (Schultz and Riggin 1985; Babich and Borenfreund 1987a, b; Cenci et al. 1987). However, using poorly differentiated cell types these studies only focussed on the general cytotoxicity and do not supply information as to the effects of chlorophenols on the functional integrity of differentiated cell systems.

In this study the potential value of primary cultured rat hepatocytes was therefore investigated as an experimental model to detect short-term effects of CP on hepatocellular function and viability. Different endpoints were selected to reveal injurious effects of the test compounds: a) xenobiotic biotransformation, b) cellular energy status and c) membrane integrity.

Materials and methods

Materials
Plastic culture dishes were purchased from Becton Dickinson (Heidelberg). Cell culture medium and antibiotics were delivered by Gibco (Paisley, Scotland). Collagenase (Type I), β-glucuronidase (type H-I, containing sulfatase activity) and insulin (from bovine pancreas) were purchased from Sigma (München). Soluble rat tail collagen (type I) was obtained from Serva (Heidelberg), and fetal calf serum from Biochrom (Berlin). Pentachlorophenol (PCP, purity >99%) was purchased from Riedel de Häen (Seelze); 2,3,4,5-tetrachlorophenol (2,3,4,5-TCP), 2,4,5-trichlorophenol (2,4,5-TCP), 2,4-dichlorophenol (2,4-DCP), and 4-monochlorophenol (4-MCP) were kindly supplied in a purified form by Prof. J. K. Seydel (Borstel). The firefly luciferin luciferase kit for the estimation of ATP was delivered by LKB (Gräfelfing). All other reagents were obtained from Merck (Darmstadt), Sigma (München), and Boehringer (Mannheim).

Cell isolation and culture conditions

Hepatocytes were obtained from male Sprague-Dawley rats (180–260 g) by a modified version of the two-step collagenase perfusion technique proposed by Seglen (1973). Rats were anaesthetized with Na-pentobarbital. The liver was perfused in situ via the portal vein with a Ca$^{2+}$-chelating Hepes buffer for 10 min (composition in mM: NaCl 145, KCl 5.4, MgSO$_4$ 0.77, MgCl$_2$ 0.93, Na$_2$HPO$_4$ 0.34, KH$_2$PO$_4$ 0.44, Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10, EGTA (ethylene glycol bis(β-aminoethoxy ether)-N,N-tetraacetic acid) 0.2; pH 7.4). The perfusion flow was kept constant at a rate of 25 ml/min by means of a roller pump. The liver was excised and trans-
ferred to a recirculating system containing 80 ml of the Hepes buffer mentioned above without EGTA, but supplemented with glucose (11 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml). Collagenase (0.06%, w/v) and CaCl2 (2 mM) were added, and the perfusion was continued for 15 min. Both perfusion buffers were continuously oxygenated and kept at a perfusion temperature of 37°C. The liver was then transferred into 4°C cold, oxygenated isolation buffer (composition in mM: NaCl 137, KCl 5.4, MgSO4 1.17, Na2HPO4 0.79, KH2PO4 0.15, Hepes 10, CaCl2 1.0, glucose 5.6), where the capsule was gently disrupted to liberate the cells. The resulting cell suspension was filtered through two layers of nylon mesh (150 and 80 μm pore diameter), submitted to low-speed centrifugation (approx. 10 g, 3.5 min) and the pellet washed twice in isolation buffer.

Finally, the cells were resuspended in Waymouth medium MB 752/1 (supplemented with fetal calf serum (10%), insulin (10⁻⁶ M), penicillin (50 U/ml) and streptomycin (50 μg/ml) to yield a final concentration of about 0.8 × 10⁶ viable cells/ml. Cell counts were performed with a hemocytometer, and cellular viability was assessed by trypan blue dye exclusion. It routinely exceeded 90%. The cell suspension was seeded onto collagen-coated culture dishes (3 ml/dish). The cultures were quickly harvested in 100 μl TRIS-EDTA-buffer (composition in mM: TRIS 20, EDTA 2, MgCl2 9, KCl 5, pH 7.5). The final concentration of acetone was 0.2% (v/v). Since acetone has been shown before to inhibit the O-deethylation of 7-ethoxycoumarin (7-EC) (about 25% inhibition at 0.2 vol%) control incubations were run at identical solvent concentrations. None of the other endpoints measured were affected by the solvent. All incubations were carried out for 1 h at 37°C. Free 7-hydroxycoumarin (7-HC) was directly measured in cells and incubation medium. For the assay of total 7-HC produced (free + conjugated metabolite) 100 μl and 400 μl aliquots of the medium and cell homogenate, respectively, were diluted to 1 ml with isotonic saline solution and incubated for 2 h at 37°C with 0.2 ml 0.2 M acetate buffer, pH 4.5, containing 1 mg crude β-glucuronidase to allow conversion of the conjugates to free 7-HC.

Leakage of lactate dehydrogenase (LDH). The effect of CP on cellular membrane integrity was assessed in the same culture dishes that were used for the EOD assay. At the end of the 1-h incubation period aliquots of the incubation buffer and cell homogenate were used for separate estimation of LDH activity (Bergmeyer and Bert 1974). LDH leakage of control and experimental cells was expressed as percentage of total LDH activity (intracellular + extracellular activity) found in the incubation medium.

Protein. Protein was measured by the method of Lowry et al. (1951).

Statistical analysis and presentation of results
Whenever three or more separate cell preparations were used, the results are given as mean ± s. e. m. The results of single preparations in all cases are derived from triplicate (EOD and LDH assay) or duplicate (ATP assay) culture dishes at each experimental condition. EC₅₀ values for the CP effects on different cellular functions are graphically determined from the mean concentration-effect curves and represent those CP concentrations which reduce the parameter in question to 50% of the respective control value.

Results
The incubation of cultured rat hepatocytes with CP for 1 h markedly impaired EOD activity as measured by the total...