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SHORT COMMUNICATIONS

Depletion of cytochrome P-450 and alterations in activities of drug metabolizing enzymes induced by cephaloridine in the rat kidney cortex

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Cephaloridine (CPH) is a cephalosporin antibiotic which induces a variety of dose-, sex-, and age-dependent nephrotoxic effects [1–3]. The most recent hypothesis on the biochemical mechanism of CPH nephrotoxicity suggests an involvement of lipid peroxidation initiated by reactive oxygen species [1]. Recent studies showed that formation of CPH-induced reactive oxygen species and peroxidation of renal cortical membrane lipids was inhibited by radical scavengers and antioxidants [4, 5]. The CPH-induced reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical could, in addition to inducing peroxidative damage of the membrane lipids [1], destroy and/or inactivate proteins [6]. The aim of the present study was to investigate the effects of CPH on the renal microsomal cytochrome P-450 and drug metabolizing enzymes.

Methods

Chemicals and biochemicals. The materials used in the present study were obtained from the following sources: cephaloridine (CPH), cytochrome *c*, 1-chloro-2,4-dinitrobenzene (CDNB), standard proteins for SDS gel electrophoresis and 7-ethoxycoumarin were purchased from Sigma Chemical Company (St Louis, MO). 7-Hydroxycoumarin was obtained from Aldrich Chemie (Steinheim, F.R.G.). Aminopyrine was obtained from Janssen Chimica (Beerse, Belgium) and aniline was purchased from Riedel-de Haen, (Seelze, Hannover, F.R.G.). All other chemicals and biochemicals were of highest reagent grade.

Animal treatment and preparation of microsomes. Male Wistar rats (Hoechst AG, Frankfurt/Main, F.R.G.) weighing 250–350 g were used. Rats were treated intravenously with 250, 500 or 1200 mg/kg/d CPH for 72 hr or with 1200 mg/kg CPH for 3, 6, 12, 24 or with 1200 mg/kg/d for 48 or 72 hr. Control rats were given the corresponding volume of the vehicle (0.9 g/100 ml NaCl). Animals were killed by cervical dislocation, kidneys were removed immediately, decapsulated and placed in ice-cold 0.9 g/100 ml NaCl. Kidney cortex was removed and microsomes were prepared from the renal cortex as described previously [7] using a phosphate buffer [8]. Sucrose was omitted from the phosphate buffer since sucrose can interfere with the assay of malondialdehyde. The present study was carried out with freshly prepared microsomes which were kept under nitrogen until used for the peroxidation studies to avoid autooxidation of the unsaturated fatty acids. Microsomal cytochrome P-450 was isolated from rat renal cortex as previously described [9].

Determination of malondialdehyde in renal cortex and renal cortical microsomes. Lipid peroxidation was monitored by measuring formation of malondialdehyde after treatment of rats with cephaloridine. Immediately after removal of the kidney, approximately 50 mg kidney cortex was homogenized in 5 ml of the thiobarbituric acid (TBA) containing reagent mixture and malondialdehyde determination was carried out as previously described [10] with the following slight modifications. Freshly prepared microsomes (300–400 μg) from the renal cortex of the control and cephaloridine-treated rats were homogenized in 5 ml of the TBA containing reagent mixture [10] and incubated for 15 min at 100°. After cooling 2 ml chloroform were added and the organic and aqueous phases were separated by centrifugation. The absorbance of the aqueous layer was measured at 535 nm using a Shimadzu 160 spectrophotometer.

Enzyme activity assays. Cytochromes P-450 and *b*5 were measured as described by Omura and Sato [11]. The fluorimetric assay of 7-hydroxycoumarin was used to measure the *O*-deethylation of 7-ethoxycoumarin [12]. The *N*-demethylation of aminopyrine was estimated by measuring the formaldehyde released by the method of Nash [13]. The hydroxylation of aniline was determined by measuring the *p*-aminophenol release [14]. NADPH-cytochrome-*c*-reductase activity was determined according to the method of Pederson *et al.* [15] using the extinction coefficient of 21 $mM^{-1} \times cm^{-1}$ [16]. Glutathione-*S*-transferase was measured as described by Habig *et al.* [17] with CDNB as substrate. Unless otherwise specified, reaction mixtures contained microsomes (0.02–2 mg/ml) and an NADPH regenerating system [18] which consisted of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.3 μmol NADP, 0.1 μmol NADPH, 4.1 μmol glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase and 163 μmol $MgCl_2$.

SDS–polyacrylamide gel electrophoresis. The polypeptide composition of the microsomal fraction was analysed by one-dimensional SDS-gel electrophoresis [19] and quantified by densitometry using a DESAGA CD 50 densitometer (DESAGA, Heidelberg, F.R.G.). Molecular weights of microsomal polypeptides were determined using a mixture of standard proteins. Protein was assayed by the method of Schacterle and Pollack [20] using bovine serum albumin as standard.

Calculations. Data were analyzed by a one-way analysis of variance. Keuls-Neuman-test was used to compare the means. Significance was set at $P < 0.05$.

Results and discussion

Treatment of rats with CPH caused an *in vivo* depletion of the microsomal cytochromes P-450 and *b*5 from renal cortex (Table 1). SDS-gel electrophoresis of renal cortical microsomes revealed a clear decrease in the extent of stained polypeptides in the molecular weight range 50–53,000 as well as an induction of polypeptide of molecular weight 44,000 (Fig. 1). In order to determine whether the polypeptides of molecular weights 50–53,000 belong to the cytochrome P-450 isoenzymes, a partial purification of cytochrome P-450 from the renal cortex of control rats was carried out [9]. The isolated cytochrome P-450 fraction had a reduced CO complex absorbance maximum at 450 nm and migrated electrophoretically in the region of the two depleted bands in the molecular weight range 50–53,000. Intraperitoneal treatment of rats for three days with 80 mg/kg/d phenobarbital, a known inducer of the cytochrome P-450, led to an increase in the intensity of the polypeptides in the 50–53,000 molecular weight region (unpublished results). These data suggest that the two depleted polypeptides of molecular weights 50–53,000 were cytochrome P-450 isoenzymes. These results are in good agreement

Table 1. Effects of cephaloridine i.v.-administration (2 d, 1200 mg/kg/d) on cytochrome P-450 and cytochrome *b*5 from rat renal cortex.

	Control rats	Treated rats	% of control
Cytochrome P-450 (nmoles/mg protein)	0.193 ± 0.004	0.033 ± 0.001*	17.1
Cytochrome *b*5 (nmoles/mg protein)	0.107 ± 0.002	0.042 ± 0.002*	40.1

Results are $\bar{x} \pm SD$ of measurements from 5 different preparations.
* Values are significant at $P < 0.05$.

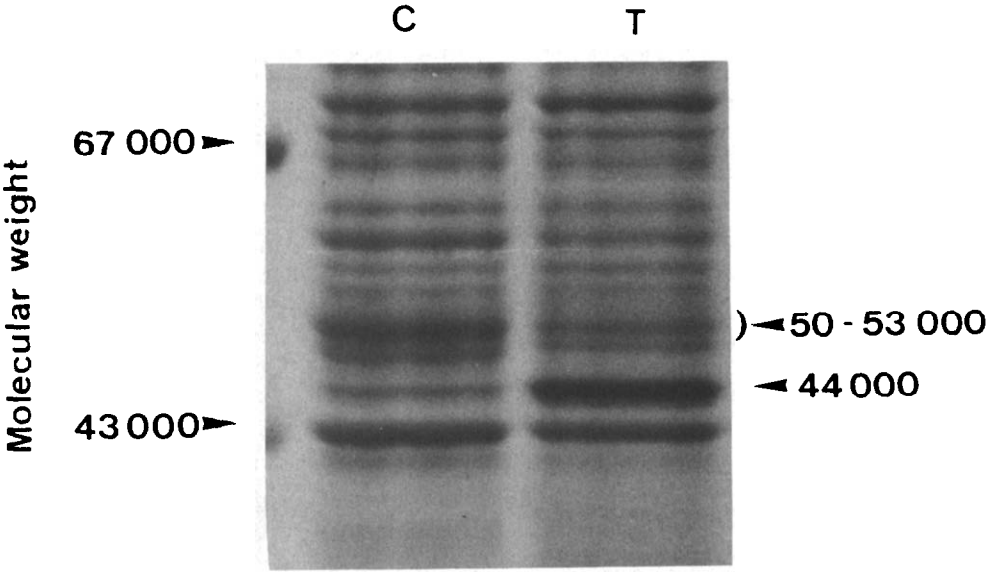

Fig. 1. Separation of microsomal polypeptides from renal cortex after SDS–gel electrophoresis. Three days after administration of CPH microsomes were prepared from the renal cortex of control and treated rats. Renal cortex microsomes were then submitted to SDS–gel electrophoresis. C, control; T, after 3 days i.v.-treatment of rats with 1200 mg/kg/d cephaloridine.

with the results of a previous study showing the presence of two forms of the renal microsomal cytochrome P-450 [21].

Renal cortical cytochrome P-450 was depleted in a dose-dependent manner (Fig. 2). A significant depletion of cytochrome P-450 occurred as early as 3 hr after a single dose of cephaloridine (Fig. 3). Twelve and 24 hr after CPH-treatment, cytochrome P-450 was almost completely depleted in the rat renal cortical microsomes. A slow recovery occurred after 48 and 72 hr despite continuing CPH-treatment. Similar results were shown for cytochrome *b*5 (data not shown). A significant increase in CPH-induced lipid peroxidation occurred in renal cortex and in renal cortical microsomes 24 hr after CPH-administration (Fig. 4). The time course of cytochrome P-450 reported herein indicates that the onset of the cytochrome P-450 loss induced by CPH precedes the CPH-induced lipid peroxidation.

After CPH-treatment, differential changes in the time course of enzymatic activities of renal cortical drug metabolizing enzymes occurred. The activity of aniline hydroxylase decreased significantly 3 hr after CPH-treatment and did not recover during the entire treatment period. The activities of NADPH-cytochrome-*c*-reductase and aminopyrine demethylase showed a slight decrease during the first 24 hr and recovered to control or higher values after 48 hr. It is of interest to note that the decrease in activity

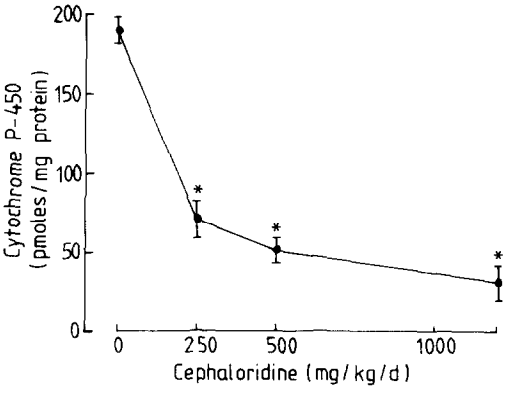

Fig. 2. Dose-dependent decrease in total microsomal cytochrome P-450 from renal cortex caused by *in vivo* i.v.-administration of cephaloridine. Following treatment of rats with CPH in different doses for 3 days, renal cortical microsomes were prepared and cytochrome P-450 was determined in microsomes from control and treated rats. Each symbol represents $\bar{x} \pm SD$ of five independent measurements. * Significantly different from the control group ($P < 0.05$).

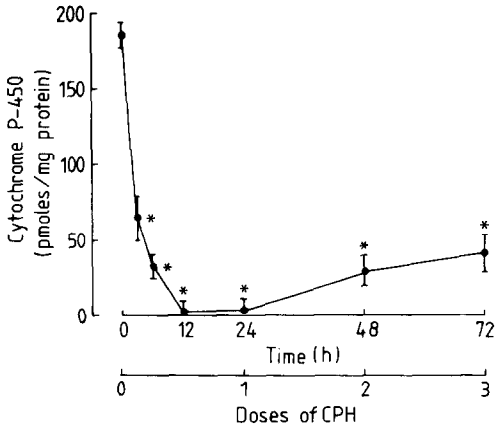

Fig. 3. Time-dependent decrease in total microsomal cytochrome P-450 from renal cortex caused by *in vivo* i.v.-administration of multiple 1200 mg/kg doses of cephaloridine. Rats were given 1200 mg/kg CPH and killed at different time points (3–72 hr) from the onset of CPH administration, and renal cortical microsomes were prepared. Subsequently, cytochrome P-450 was determined spectrophotometrically in renal cortical microsomes from control and treated rats. Each symbol represents $\bar{x} \pm SD$ of five independent measurements. * Significantly different from the control group ($P < 0.05$).

of some drug metabolizing enzymes such as aniline hydroxylase paralleled the loss in cytochrome P-450 and occurred in the absence of a detectable lipid peroxidation during the first 12 hr after CPH-administration. However, the aminopyrine-*N*-demethylase remained unaltered although in the treated rats the amount of cytochrome P-450 decreased significantly. At the same time the enzymatic activities of 7-ethoxycoumarin-*O*-deethylase and glutathione-*S*-transferase increased significantly when compared to controls. Glutathione-*S*-transferase showed a threefold increase in activity after 48 hr in the presence of a simultaneous depletion of cytochrome P-450 (Table 2). These findings speak for highly selective effects of the CPH treatment on the activity of various drug metabolizing enzymes. A recent *in vitro* study [22] has shown that CPH inhibits the activity of the glucose-6-phosphatase from the renal cortical microsomes. The ability of CPH to cause alterations in the activity of other enzymes than drug metabolizing enzymes suggests that CPH induces changes in the activity of renal cell enzymes in an unspecific manner.

Xenobiotics such as carbon tetrachloride [23], trichloroethylene [24] or cadmium [25] have been shown to destroy cytochrome P-450, to inhibit and/or induce the activity of various drug metabolizing enzymes in a manner similar to that of CPH. The mechanism of the specific cytochrome P-450 loss and inactivation of drug metabolizing enzymes induced by CPH may be due either to a direct attack of radicals on proteins or to initiation of the peroxidation of microsomal lipids. Subsequently, these primary biochemical lesions could result in damage to the cytochrome P-450 and inactivation of drug metabolizing enzymes. Since CPH induced the formation of reactive oxygen species [1], it is likely that these oxygen radicals are responsible for the *in vivo* depletion of cytochrome P-450 and the inactivation of drug metabolizing enzymes.

In conclusion treatment of rats with the cephalosporin antibiotic cephaloridine resulted in a dose- and time-dependent depletion of renal microsomal cytochromes P-450, *b*5, and peroxidation of membrane lipids. A slow recovery of the depleted renal cortical cytochrome P-450 occurred at 48 and 72 hr despite continuing cephaloridine treatment. Additionally, cephaloridine treatment selectively altered the enzymatic activity of various drug metabolizing enzymes.

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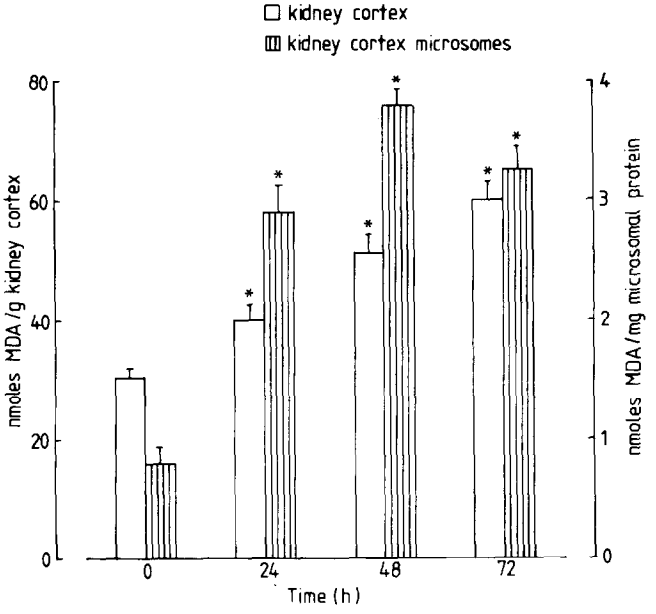

Fig. 4. Time-dependence of cephaloridine-induced lipid peroxidation *in vivo*. After i.v.-administration of CPH (1200 mg/kg/d) for 12, 24, 48 and 72 hr, malondialdehyde (MDA) was determined in kidney cortex and renal cortical microsomes. Values are $\bar{x} \pm SD$ of five independent measurements. * Significantly different from the control group ($P < 0.05$).

Table 2. Effects of cephaloridine i.v.-administration (2 d, 1200mg/kg/d) on the activity of some drug metabolizing microsomal enzymes from rat renal cortex

	Control rats	Treated rats	% of control
NADPH-cytochrome-c-reductase (nmoles/mg protein/min)	13.64 ± 2.81	11.65 ± 2.03	85.2
Aminopyrine-*N*-demethylase (nmoles/mg protein/min)	0.81 ± 0.12	0.82 ± 0.07	101.2
Aniline hydroxylase (nmoles/mg protein/min)	0.330 ± 0.02	0.018 ± 0.003*	5.5
7-Ethoxycoumarine-*O*-deethylase (nmoles/mg protein/min)	0.095 ± 0.02	0.130 ± 0.07*	136.8
Glutathione-S-transferase (nmoles/mg protein/min)			
I. Microsomal	9.8 ± 1.6	35.0 ± 1.8*	357.1
II. Cytosolic	94.75 ± 7.4	298.62 ± 11.3*	315.2

Results are $\bar{x}$ ± SD of measurements from 5 different preparations.
* Values are significant at $P < 0.05$.

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Phenothiazine-mediated depolarization of the plasma membrane in a renal cell line

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The phenothiazines have been described recently as agents useful for the protection of ischemic tissue [1–4]. Several authors, however, have commented upon the cellular toxicity of phenothiazines, describing increased plasma membrane permeability [2], plasma membrane blebbing [2], mitochondrial abnormalities [2, 5], and decreased cellular proliferation and clonogenicity [6]. Here, we report phenothiazine-induced depolarization of the plasma membrane after exposure to low concentrations of phenothiazines. This depolarization represents an early effect of phenothiazines on the cell membrane.

Materials and methods

Cell culture. LLC-PK1 renal epithelial cells (between passages 209 and 230) were cultured according to methods previously described [2]. The culture medium consisted of Dulbecco's Minimal Essential Medium (DMEM) containing glutamine and 10% fetal bovine serum. Cells were grown at 37° in a humid 5% carbon dioxide atmosphere until utilized in experiments, which were performed at room temperature (20°).

Reagents and incubation techniques. Promethazine, chlorpromazine, trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-napthalene sulfonamide (W-7) were obtained from Sigma. 1,1',3,3'-Hexamethylindocarbocyanine iodide (DiICl (3)) was obtained from Molecular Probes (Junction City, OR).

For all experiments, approximately 10^6 cells were incubated in 2 ml of Earle's Balanced Salt Solution (EBSS) containing 4 g/L glucose and either 50 μM or 1.8 mM $CaCl_2$ at pH 7.3. Plasma membrane potential was quantitated using the fluorescent probe DiICl(3). This is a cationic fluorescent probe which partitions between the cytoplasm and the extracellular space in accordance with the magnitude of the membrane potential [7, 8]. A stock solution of this dye in dimethyl sulfoxide (2 mM) was stored at 0° and diluted to 1 μM in EBSS prior to use. An equilibrium distribution, as demonstrated by stable cellular fluorescence, was achieved after 15-min incubations of the cells in the dye solution. Phenothiazines were added to the suspension at concentrations varying from 0 to 100 μM, and measurements were made after 30-min exposures of the cells to the phenothiazine.

Cells were harvested using one of two methods in order to produce populations of cells with differing basal membrane potentials: (1) exposure of monolayers to a 0.25% trypsin–EDTA solution at 37° until detachment from substratum, and (2) sequential exposure of monolayers to Ca^{2+}-free EBSS solution at 37° for 30 min followed by exposure to Ca^{2+}-free EBSS solution containing 6 units/ml papain, again at 37° for 5–10 min until detachment.

Flow cytometry. Flow cytometric analysis was performed with an FACS-III fluorescent-activated cell sorter (Becton-Dickenson) using a 488 nm laser operated at 300 mW. Scatter size was set to bracket the LLC-PK1 cell size distribution while excluding both debris and cellular clumps from measurement. A bandpass filter was placed in front of the photomultiplier tube to ensure that only fluorescent light was recorded. Relative membrane potential was measured as described previously [7, 8]. Membrane potential recordings were begun at least 15 min after addition of the fluorescent probe to the cell suspension, at a time when a constant level of dye accumulation had been observed in the cells, indicating equilibrium.

Statistical analysis. All statistical analyses were performed using analysis of variance, followed by the two-tailed *t*-test for unpaired data when the F-test indicated a significant difference between groups. Bonferroni's correction was used, when appropriate, to correct for multiple comparisons.

Results

Figure 1 shows the effect of various concentrations of phenothiazines on the plasma membrane potential in LLC-PK1 cells, measured after 30-min exposures to the phenothiazine. The mean concentrations required to depolarize the plasma membrane to 50% of its control value (IC_{50}) were 9 μ3 μM for trifluoperazine, 19 ± 2 μM for chlorpromazine ($P < 0.05$ from trifluoperazine), 39 ± 2 μM

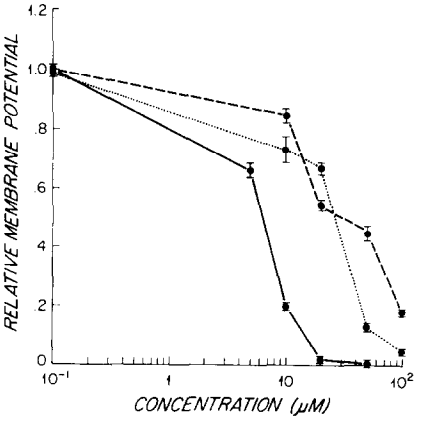

Fig. 1. Effect of various concentrations of phenothiazines upon plasma membrane potential. Relative membrane potential indicates that percent dye content of cells after 30 min of phenothiazine exposure relative to the dye content of control cells at 30 min. Points are means ± SEM, N = 4. Each curve is representative of several experiments. Key: (——) trifluoperazine, (.....) chlorpromazine, and (— —) promethazine.