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Celiptium-induced nephrotoxicity and lipid peroxidation in rat renal cortex

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Summary. The antitumor drug celiptium, or N2-methyl-9-hydroxyellipticinium (NMHE), is an ellipticine derivative used in the treatment of breast cancer. Celiptium-induced dose-dependent renal toxicity in rats is characterized by tubular necrosis, tubulo-interstitial lesions and lipid overload in proximal tubular cells. Since biooxidative activation of celiptium occurs in kidney via highly electrophilic intermediates, we studied the effects of celiptium on rat renal cortex lipids in the context of lipid peroxidation damage. Female Wistar rats were injected with a single i.v. dose of 20 mg/kg celiptium and were killed on day 2, 4 or 8. Histochemical analysis of kidney sections detected Oil Red O (ORO)-positive deposits, whereas the same sections studied using Holczinger's copper rubeanic acid method showed free fatty acid (FFA) granules in renal tubular cells of celiptium-treated rats. Electron microscopy revealed large fatty droplets in proximal tubular cells. As creatinine clearance decreased on days 4 and 8, celiptium induced a significant increase in renal cortex FFA levels (6-fold increase over pretreatment values on day 8), whereas total glycerides increased 1.5 times. A 15% decrease in total phospholipids (PL) and a 50% decline in the mass of phosphatidylethanolamine (PE) were detected by lipid phosphorus assay. A 1.2-fold decrease in the unsaturation index of total PL was noted, with a significant decline in arachidonic acid (20:4). A 15% decrease in arachidonic content was observed in the fatty acid composition of PE. Analysis of the fatty acid composition of neutral lipids showed changes only in the FFA class. A great proportion of oleic (18:1) and linoleic (18:2) acids was found. Iodometric titration and thiobarbituric acid (TBA) reactivity detected respectively significant amounts of lipid hydroperoxides and TBA-reactive material in renal cortex lipid extracts on days 2, 4 and 8. The lipid-peroxidation process appeared to be involved in the pathogenesis of celiptium nephrotoxicity.

Introduction

The antitumor drug celiptium (N2-methyl-9-hydroxyellipticinium, NMHE; Fig. 1) is an intercalating agent of the alkaloid family. Celiptium is an ellipticine derivative that is effective in the treatment of experimental tumors [31] and is used especially to treat bone metastases of human breast carcinomas [17]. Celiptium has been demonstrated to have higher in vitro activity than Adriamycin against breast cancer, renal cell carcinoma and non-small-cell as well as small-cell lung cancer [1]. Among the most important characteristics of this drug is its lack of myelotoxicity and hepatotoxicity; nevertheless, nephrotoxicity has been shown in man [16, 33]. We recently described dose-dependent renal toxicity after a single dose of celiptium in the Wistar rat [32]. This toxicity is characterized by acute renal failure, with tubular necrosis and subsequent tubular interstitial lesions occurring 60 days after drug administration.

Recently, Monsarrat et al. [27] showed that the main compound excreted in human and rat urine after celiptium treatment was unchanged drug accompanied by S-conjugates, proving indirectly the presence of an "in vivo oxidized intermediate form", which is a quinone-imine N2-methyl-9-oxoellipticinium (NMOE). Moreover, it was shown that isolated rat kidney cells metabolize celiptium into *N*-acetylcysteinyl or cysteinyl conjugates, the same S-conjugates detected in both rat and human urine, whereas these compounds were not found in bile [23, 24]. An in vitro study [2] has demonstrated autooxidation of hydroxy

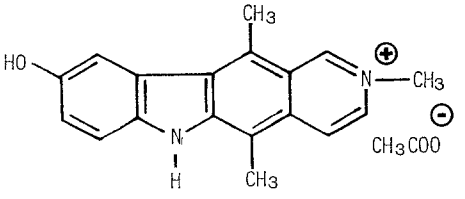

Fig. 1. Chemical structure of N2-methyl-9-hydroxyellipticinium (NMHE)

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derivatives of ellipticine, leading to the formation of quinone-imine derivatives. Thus, biooxidative activation of celiptium occurs in the kidney and results in the formation of highly electrophilic intermediates. We tried to determine whether such reactive metabolites induce intrarenal lipid-peroxidation damage and therefore decided to investigate the effects of celiptium on renal cortex lipids.

Materials and methods

Female Wistar rats weighing approximately 200 g and given access to drink and food received celiptium or NMHE by intravenous injection in a tail vein. Celiptium was provided by the Sanofi Company (batch RD 6792) and was dissolved in 0.02 ml 5% glucose/100 g body weight. A total of 18 rats received a single i.v. dose of 20 mg/kg celiptium (equivalent to 60 mg/m^2), and 6 control rats received the same volume of 5% glucose. Animals were killed at 2, 4 or 8 days after drug administration. Rats were acclimated to metabolic cages for a 48-h period before being killed. Urine was collected for two consecutive 24-h periods. At the time of sacrifice, animals were anesthetized intraperitoneally with Nesdonal (4 mg/100 g body weight) and a catheter was placed in the abdominal aorta for blood sampling and perfusion of the kidneys with 100 m*M* saline phosphate buffer (pH 7.4); 2 or 3 ml blood was collected in a dry tube.

Morphological techniques

Light microscopy. Fine sections (2 mm) of kidney were fixed by immersion in Duboscq-Brasil fluid (75 ml alcohol, 30 ml formol, 7.5 ml acetic acid, 0.5 g picric acid) for 5 h. After dehydration with absolute alcohol, specimens were embedded in paraffin and 3-μm sections were cut and stained with hematoxylin/eosin and periodic acid-Schiff stain.

Histochemistry. Sections of kidney were immediately snap-frozen in liquid nitrogen and processed for histochemistry. Unfixed frozen sections (5 μm) were prepared with a cryostat (Leitz-1720 Digital). Neutral lipids were demonstrated using Oil Red O (ORO) [4]. Unsaturated hydrophobic lipids were stained red and phospholipids, pink. The same cryostat sections were post-fixed in formalin-calcium and studied using the copper rubeanic acid method [12], by which free fatty acids are stained dark green. Control material included delipidised sections (acetone-extracted) for verification of the lipid nature of the stained reaction product.

Electron microscopy. Small pieces of kidney (1 mm^3) were dissected and fixed by immersion for 1 h in 2.5% glutaraldehyde in 5% sucrose-0.1 M cacodylate buffer (pH 7.3) and then post-fixed in 2% osmium tetroxide. After embedding in epoxy resin (Glycidether 100, Merck), ultrathin sections were cut with a Reichert OM-U2 ultramicrotome and stained with uranyl acetate and lead citrate for examination under a Philips CM 10 microscope.

Biochemical studies

Urinary and serum creatinine levels were measured by the Jaffe reaction using a Technicon autoanalyser [7]. Kidney cortices were dissected and kept frozen at –80° C until analysis. Homogenates were made in distilled water (1 : 50, w/v) with a Kontess conical, sintered-glass tissue grinder. Proteins were measured by the method of Lowry et al. [21] using bovine albumin as a standard. Lipids were extracted from 1-ml aliquots of renal cortex homogenates by the method of Bligh and Dyer [5].

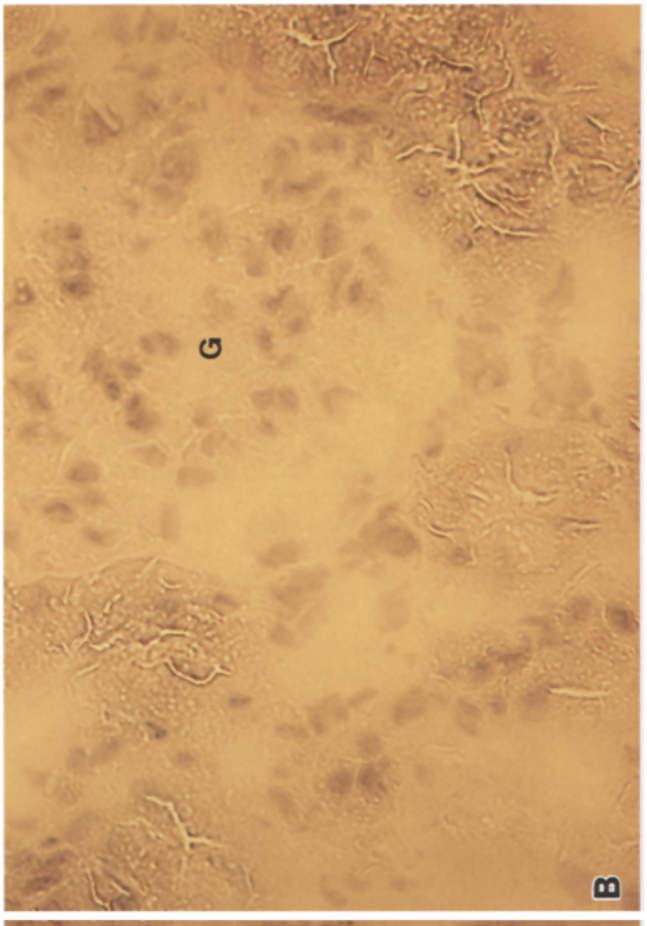
Lipid analysis. Phospholipids (PL) were resolved for fatty acid analysis by high-performance liquid chromatography (HPLC) according to Shi-Hua Chen and Kou [34]. Analysis was performed on a Beckman liquid chromatograph consisting of a model 112 Beckman delivery system, a model 340 injector, a 421 Programator C model 160, a 214-nm wavelength detector and a Shimadzu integrator (CR3A). The chromatographic column was 25 cm × 4.6 mm (Ultrasphere Si, Beckman, with silica gel; particle size, 5 μm). The acetonitrile/methanol/85% orthophosphoric acid (130 : 5 : 1.5, by vol) solvent was delivered to the column at a flow rate of 1.5 ml/min and a pressure of 1,730 psi. Fractions were collected with a Gilson TDC 80 microcollector. PL were identified by their retention times relative to those of standards (Sigma). Cardiolipin (DPG) and neutral lipids co-eluted with the solvent front. The four major phospholipid components resolved were phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Each phospholipid was washed to remove orthophosphoric acid, dried under nitrogen and kept at –80° C until gas liquid chromatographic analysis (GLC).

Quantitation of PL could not be performed using our HPLC procedure since the solvent contains orthophosphoric acid, which interferes with phosphate measurement. Thus, for quantitative analysis, PL were resolved by one-dimensional thin-layer chromatography (TLC) on 0.25-mm HPTLC silica gel 60 plates (Merck) [38]. The solvent was chloroform/methanol/acetic acid/water (55 : 35 : 3 : 2, by vol). After development, TLC plates were visualized with iodine vapors and identification of lipid spots was confirmed by co-migration with standards (Sigma). Six major phospholipids were obtained: PC, PS, PI, PE, DPG and sphingomyelin (SPH). PL classes were quantitated by lipid phosphorus analysis according to Bartlett [3]. Aliquots of renal cortex extracts were assayed for total phosphorus. Data were expressed as nanomoles of lipid phosphorus per milligram of protein.

For quantitative analysis neutral lipids free fatty acids (FFA) were extracted using petroleum ether and glycerides and total cholesterol (cholesterol + cholesterol esters) was extracted using chloroform/methanol (2 : 1, v/v); these were assayed enzymatically with bio-Merieux and Biolyon kits. Data were expressed as nanomoles of lipid per milligram of protein. For fatty acid analysis, neutral lipids were resolved from phospholipids by silicic acid column chromatography. Triglycerides (TG), FFA and cholesterol esters were separated by TLC (0.5-mm plates, silica gel 60, Merck), developed twice in petroleum ether/diethylether/acetic acid (80 : 20 : 1, by vol) solvent. Spots were identified by co-chromatography with standards and were visualized by brief exposure to iodine vapors, scraped from plates, eluted first with diethylether and then with chloroform/methanol, and evaporated under N_2. The nonesterified fatty acid class, extracted by petroleum ether, was prepared by treatment with ethereal diazomethane because of high FFA lability in TLC [20].

GLC analysis. The fatty acid composition of each PL and each neutral lipid was analyzed by GLC after direct transesterification to the fatty acid methyl esters (FAME) using 14% boron trifluoride in methanol at 75° C for 5 min according to Morrison and Smith [29]. The analyses were carried out on a Perkin Elmer 8500 gas chromatograph equipped with a flame ionization detector using a 50% polyphenyl/50% cyanopropylmethylsiloxane column (glass column, RSL 500, Alltech). FAME were identified by their retention times relative to those of standards (Sigma). The amount of each ester was determined by a Shimadzu integrator (CR3 A). Fatty acids quantified included palmitic (16 : 0), stearic (18 : 0), oleic (18 : 1), linoleic (18 : 2), arachidonic (20 : 4) and docosahexaenoic (22 : 6) acids. Fatty acid compositions were presented as weight percentages of total FAME.

Lipid peroxide and malondialdehyde measurements. An aliquot of total lipid extract was dried under N_2, redissolved in 1 ml chloroform/methanol (2 : 1, v/v) and assayed iodometrically according to Buege and Aust [6]. Absorbance at 353 nm was determined and was converted to hydroperoxide equivalents using an absorption coefficient of 1.73×10^4 M^{-1} cm^{-1}. The thiobarbituric acid assay was performed according to the method of Ohkawa et al. [30] using chloroform/methanol (2 : 1, v/v) renal cortical extracts. After the addition of 2.5 ml thiobarbituric acid (TBA) reagent (0.6% in 15% acetic acid, pH 3.5), the mixture was heated for 15 min in boiling water and then cooled and the absorbance measured at 532 nm was converted to nanomoles of malondialde-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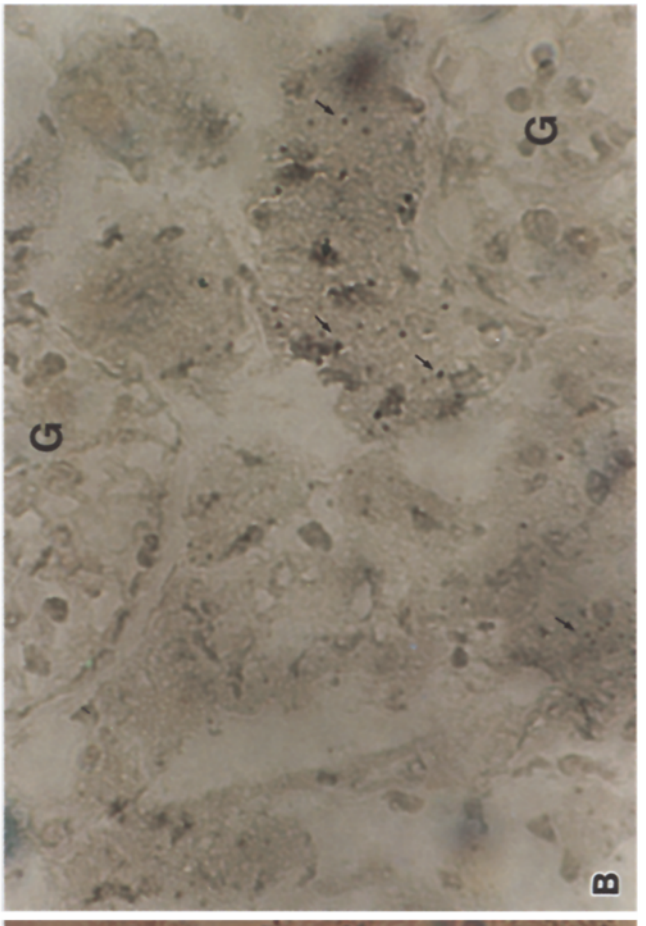
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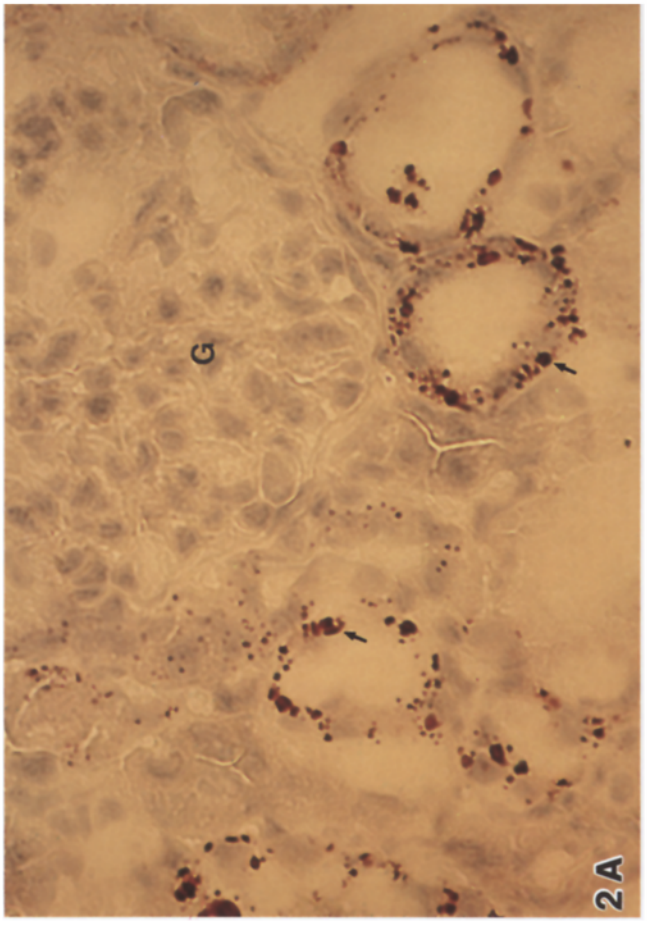
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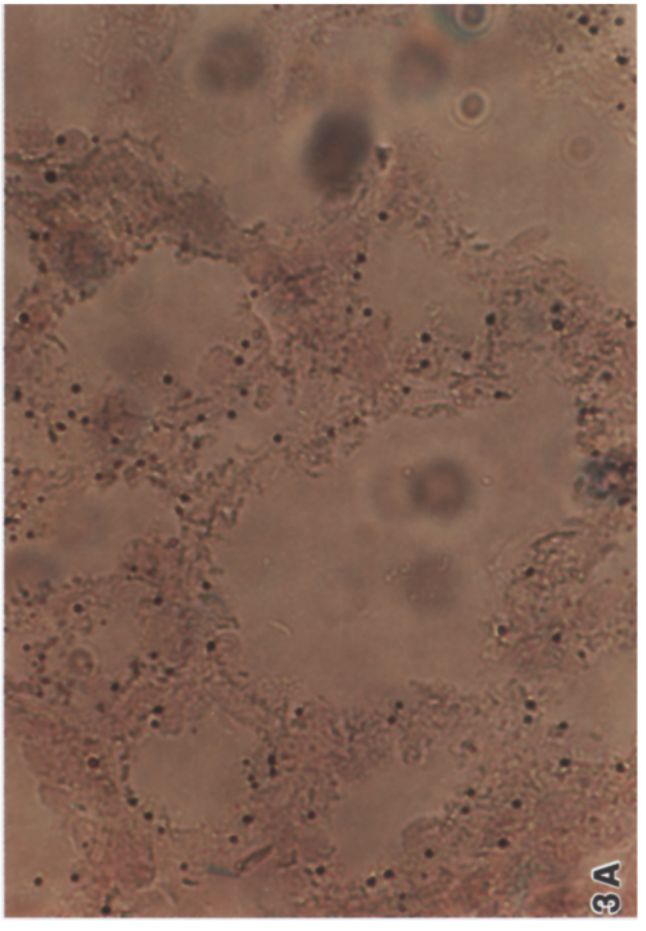
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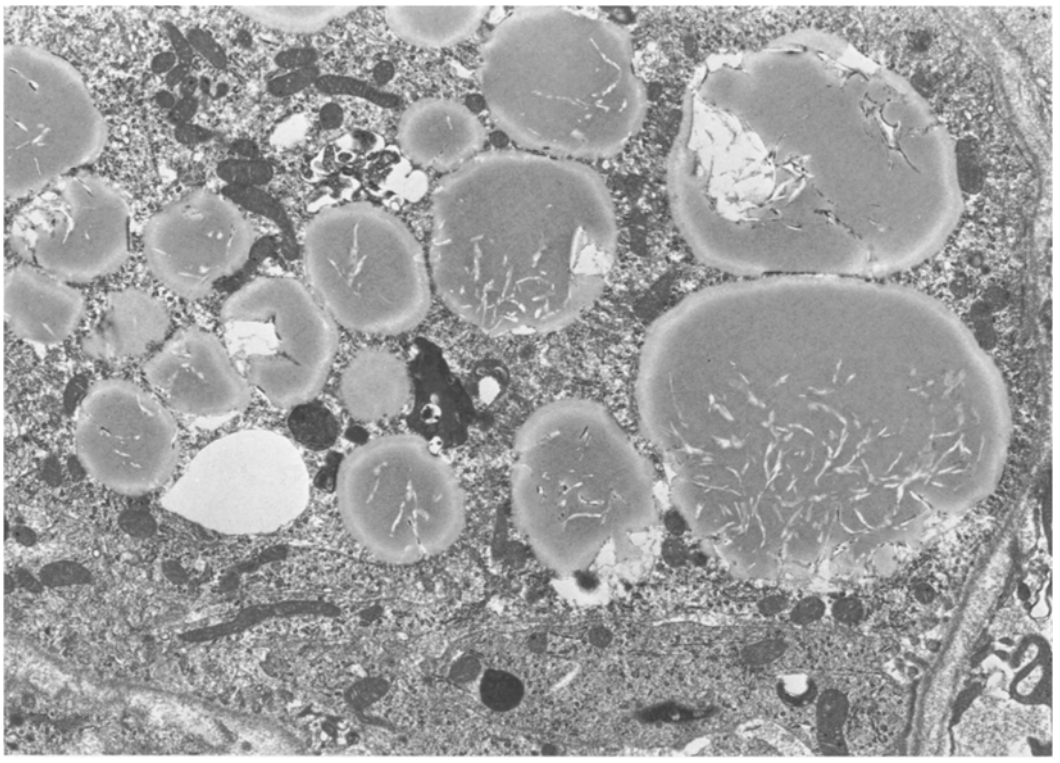

Fig. 4. Electron micrograph of a proximal tubular cell from a rat treated with 20 mg/kg celiptium on day 8. Numerous intracytoplasmic lipid deposits may be observed. Uranyl acetate and lead citrate; × 8,000

hyde (MDA) from a standard curve generated with 1,1,3,3-tetramethoxypropane (1.2 mM stock solution in 0.01 *N* HCl). Data were expressed as microequivalents of lipid peroxides or as nanomoles of MDA per milligram of protein.

Statistical analysis. Statistical analysis was performed using Student's *t*-test for control and treated values for six independent experiments. Probability values of $P < 0.05$ (*), P 0.01 (**) and $P < 0.001$ (***) were considered to be significant. Results were expressed as means ± SD.

Results

Morphology

Light microscopy was conducted as a control parallel to histochemistry. As we have previously described [32], a single dose of 20 mg/kg celiptium induced the presence of focal renal lesions, with extensive cytoplasmic vacuolization, tubular dilatation and tubular epithelial necrosis being observed on days 4 and 8. No glomerular or interstitial lesions were seen.

Histochemical analysis of kidney sections detected Oil Red O (ORO)-positive deposits in the renal cortex of each celiptium-treated rat as early as on day 2. However, on days 4 and 8 these deposits were more numerous and almost filled the renal cortex (Fig. 2A). Such red-stained deposits were seen only in proximal tubules, not in glomeruli. Control kidneys contained no lipid material in either tubules or glomeruli (Fig. 2B). The same sections studied using Holczinger's copper rubeanic acid method detected numerous FFA granules in proximal tubular cells on days 2, 4 and 8. These granules stained dark green. They appeared to be small and were dispersed in the proximal tubules as shown in Figs. 3A and 3B. In acetone-extracted sections, no characteristic ORO- or FFA-positive deposits were seen in control or treated kidneys.

Electron microscopy showed no glomerular lesions in celiptium-treated rats, whereas the presence of lipid-like deposits was noted in the cytoplasm of proximal tubular cells. No alterations were seen in the brush border, mitochondria, lysosomes or nuclei [32]. Figure 4 shows the extent to which these large fatty deposits overloaded the cells.

Fig. 2 A, B. A Light micrograph of rat renal cortex after treatment with 20 mg/kg celiptium on day 8. Accumulation of unsaturated hydrophobic lipids (*arrow*) can be seen only in proximal tubules. Cryostat section stained with ORO and counterstained with Mayer's haemalun. *G*, glomerulus. × 400. **B** Light micrograph of renal cortex from control rats. Sections stained with ORO and Mayer's haemalun. Note the absence of lipid deposits. *G*, glomerulus. × 400

Fig. 3 A, B. A Light micrograph of renal cortex from rats treated with 20 mg/kg celiptium on day 8. Cryostat section stained using Holczinger's copper rubeanic acid technique and counterstained with carmalum. Granules of free fatty acids can be seen in proximal tubules. × 400. **B** No such granules are visible in glomeruli (*G*). × 400

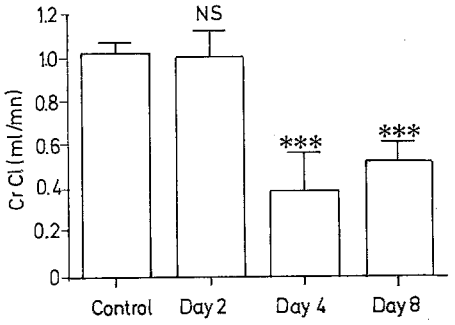

Fig. 5. Renal function in control animals and in celiptium-treated rats at 2, 4 or 8 days following a single dose of 20 mg/kg, shown as the means of 6 independent experiments. *Bar,* SD; *NS,* not significant. *** $P < 0.001$)

Table 1. Mean absolute values for individual neutral lipids of renal cortex in the control group and in treated rats after 2, 4, or 8 days following a single dose of 20 mg/kg celiptium

	Control	Day 2	Day 4	Day 8
Glycerides (nmol/mg protein)	10.4±1.5	12.3±1.8	13.7±2.9*	16.9± 3.1**
Total cholesterol	30.0±2.5	45.2±5.1***	32.4±4.5	42.0± 8.8**
Free fatty acids (nmol/mg protein)	12.5±2.3	23.7±4.5***	23.5±3.5***	73.9±10.7***

Values represent the means ±SD of 6 independent experiments.
* $P < 0.05$; ** $P < 0.001$; *** $P < 0.001$ vs control

Biochemical studies

Renal function. Creatinine clearance had decreased significantly by days 4 and 8 (Fig. 5).

Lipid mass. A single dose of 20 mg/kg celiptium induced an increase in total neutral lipid mass, whereas total phospholipid mass was decreased (Tables 1, 2). Glyceride content increased significantly after 4 and 8 days of treatment, measuring 13.7±2.9 ($P < 0.05$) and 16.9±3.1 ($P < 0.01$) nmol/mg protein respectively, vs 10.4±1.5 in the control group (Table 1). A similar increase in cholesterol content was found. The rise in FFA content was evident as early as on day 2 (23.7±4.5 vs 12.5±2.3 nmol/mg protein in controls; $P < 0.001$) and was higher on day 8 (73.9±10.7 nmol/mg protein, a 6-fold increase) than was the increase in glyceride or cholesterol content (Table 1).

Conversely, the PL content of the renal cortex showed a 15% decrease on days 4 and 8 (246.4±20.1 nmol/mg protein on day 8 vs 273.3±15.5 nmol/mg protein in controls; $P < 0.05$). Table 2 shows the percentage of individual PL analyzed after resolution by TLC. The recovery of lipid phosphorus from the plates was 93.2%±4.6% for control and treated rats. Analysis of individual PL showed a decline in only the mass of phosphatidylethanolamine (PE, 40.6±5.8 nmol/mg protein on day 8 vs 81.6±5.2 nmol/mg protein in controls; $P < 0.001$).

Measurement of lipid peroxidation. The loss of polyunsaturated fatty acid reflects the peroxidation of fatty acids. Table 3 shows that the unsaturation index decreased on days 4 and 8 for total phospholipids: 171.6±10.8 in controls vs 148.9±18.0 ($P < 0.01$) on day 8. At the same time, celiptium induced a significant increase in both lipid hydroperoxides and thiobarbituric acid (TBA)-reactive substances on days 2, 4 and 8. Hydroperoxide values increased significantly from day 2 to day 8 (23.88±8.44 μEq/mg protein on day 8 vs 2.58±1.82 μEq/mg protein in controls; $P < 0.001$), whereas values for TBA-reactive substances plateaued during this period (5.39±0.88 nmol MDA/mg protein on day 8 vs 3.05±0.48 nmol MDA/mg protein in controls, $P < 0.001$).

Fatty acid analysis of the renal cortical PL subclasses. Table 4 shows the fatty acid content of PL in control and treated rats on day 8. Significant changes took place in total

Table 2. Phospholipid content expressed as a percentage of total phospholipid and as nmol P_i/mg protein in renal cortex of control animals and of rats treated with 20 mg/kg celiptium at various intervals

	Control	Day 2	Day 4	Day 8
Total phosholipids (nmol P_i/mg protein)	273.3±15.5	237.1±38.0	233.2±16.6**	246.4±20.1*
PC (%)	30.8± 1.6	32.4± 1.8	33.0± 3.3	34.4± 2.3
(nmol P_i/mg protein)	84.1± 4.7	76.9±13.4	77.2±11.3	84.9± 9.6
PE (%)	29.9± 2.7	23.5± 3.6	18.0± 2.6	18.7± 5.8
(nmol P_i/mg protein)	81.6± 5.2	56.0±14.9**	42.0± 6.3***	40.6± 5.8**
PI+PS (%)	12.5± 2.3	12.3± 1.5	17.4± 3.5	17.0± 2.6
(nmol P_i/mg protein)	34.2± 7.2	29.1± 5.1	40.4± 6.4	41.8± 7.4
SPH (%)	17.0± 2.4	19.5± 1.9	22.9± 4.6	20.1± 4.6
(nmol P_i/mg protein)	46.4± 7.8	46.2± 8.5	53.6±12.1	49.7±13.2
DPG (%)	7.9± 1.9	11.0± 2.7	9.2± 2.1	9.4± 1.4
(nmol P_i/mg protein)	21.6± 5.8	25.7± 5.9	21.3± 4.8	3.2± 3.9

Values represent the means ±SD of 6 independent experiments. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; DPG, diphosphatidylglycerol. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs control

Table 3. Index of lipid peroxidation measured by unsaturation indices for fatty acids of renal cortical phospholipids, by iodometric titration of lipid hydroperoxides and by thiobarbituric acid reactivity

	Control	Day 2	Day 4	Day 8
Phospholipids (unsaturation index)[2]	171.6 ±10.8	161.8 ±15.2	146.3 ±12.2***	148.1 ±18.0**
Lipid hydroperoxides (μEq/mg protein)	2.58± 1.82	8.86± 6.4**	20.56± 2.59***	23.88± 8.44***
TBA reactivity (nmol MDA/mg protein)	3.05± 0.48	5.27± 0.67***	5.39± 0.58***	5.33± 0.88***

Values represent the means ±SD of 6 independent experiments. $P<0.05$; ** $P<0.01$; *** $P<0.001$ vs control
[a] Unsaturation index calculated as (number of double bonds × area %

Table 4. Major fatty acid composition of total phospholipids and of each subclass in renal cortex from control animals or rats treated with 20 mg/kg celiptium on day 8.

Fatty acid	PL		PE		PC		PI		PS	
	Control ($n=6$)	Day 8 ($n=6$)	Control ($n=6$)	Day 8 ($n=5$)	Control ($n=6$)	Day 8 ($n=5$)	Control ($n=6$)	Day 8 ($n=5$)	Control ($n=6$)	Day 8 ($n=5$)
16:0	24.8 ±2.8	27.3 ±4.8	21.0 ±4.8	23.7 ±2.8	36.7 ±3.0	38.4 ±1.0	30.8 ±6.2	30.4 ±8.0	28.3 ±2.5	28.2 5.2
18:0	17.2 ±3.0	18.5 ±3.1	29.6 ±4.3	30.5 ±2.1	18.6 ±2.2	17.7 ±1.5	32.0 ±3.4	31.4 ±8.5	37.7 ±5.3	37.3 ±8.0
18:1	11.4 ±1.7	13.5 ±0.9	9.7 ±0.8	11.4 ±0.7	10.3 ±1.0	11.7 ±1.3	11.9 ±2.4	13.0 ±3.3	11.4 ±2.8	10.1 ±8.5
18:2	16.0 ±0.5	16.4 ±2.5	6.4 ±1.5	6.6 ±0.7	10.6 ±1.4	12.6 ±1.9	13.2 ±4.5	12.2 ±3.9	6.9 ±1.1	7.3 ±0.7
20:4	27.7 ±1.6	21.6 ±2.7	30.9 ±3.0	26.3 ±3.7	21.4 ±4.4	17.8 ±3.7	10.9 ±2.6	11.8 ±1.9	14.3 ±3.4	15.9 ±3.2
22:6	2.9 ±0.8	2.7 ±1.3	2.4 ±0.6	1.5 ±1.1	2.4 ±1.4	1.8 ±1.4	1.2 ±0.5	1.2 ±1.1	1.4 ±1.2	1.2 ±1.2
Unsaturation index[a]	171.6 ±10.8	148.9 18.0**	160.5 ±11.7	138.8 ±16.0	131.5 ±19.4	118.9 ±12.0	89.1 ±15.3	91.8 ±9.0	90.8 ±16.7	95.5 ±16.1

Values represent the mean (±SD) area %. PL, total renal cortex phospholipids. Other abbreviations as shown in Table 2. **, $P<0.01$ vs control
[a] Unsaturation index calculated as (number of double bonds × area %)
C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C20:4, arachidonic acid; C22:6, docosahexaenoic acid;

Table 5. Major fatty acid composition of triglycerides, free fatty acids and cholesterol esters in control animals or rats treated with 20 mg/kg celiptium on day 8. Esterification of FFA was performed with diazomethane; esterification of TG and cholesterol esters resolved using the TLC technique was done in the classic manner

Fatty acid	FFA		TG		Cholesterol esters	
	Control ($n=6$)	Day 8 ($n=6$)	Control ($n=6$)	Day 8 ($n=6$)	Control ($n=6$)	Day 8 ($n=6$)
16:0	23.5± 3.9	19.4± 2.5	49.3±6.3	55.5± 7.4	44.1±2.5	42.5± 5.5
18:0	8.1± 2.9	5.1± 0.4	14.1±2.0	11.9± 3.2	21.6±2.9	22.6± 4.8
18:1	9.4± 1.5	13.4± 2.7	19.8±4.4	17.3± 3.6	22.8±2.6	23.0± 3.8
18:2	19.1± 2.1	23.5± 2.0	14.6±2.9	13.3± 2.8	9.6±2.0	9.6± 1.5
20:4	38.7± 4.4	37.9± 5.0	1.8±0.6	1.7± 0.9	1.4±0.3	1.8± 0.9
22:6	1.2± 0.7	0.7± 0.3	0.4±0.2	0.3± 0.2	0.5±0.2	0.4± 0.11
Unsaturation index[a]	209.6±16.4	227.2±13.2*	58.6±9.5	52.5±14.1	50.6±8.6	51.8±10.5

Values represent the mean (±SD) area % of 6 independent experiments. Abbreviations as shown in Table 2. * $P<0.05$ vs control
[a] Unsaturation index calculated as (number of double bonds × area %)

PL of rat renal cortex, with a decrease in arachidonic acid content occurring in treated rats (27.2% ± 1.6% in controls vs 21.6% ± 2.7% on day 8).

The major PL components of the renal cortex were separated by an HPLC procedure that offers an easy and reproducible method for collection and avoids deterioration of samples that are to be subsequently analyzed by GLC [47]. The fatty acid composition of total PL was the same before and after the HPLC procedure (PL fractions were collected and pooled; the percentage of recovery of

each fatty acid ranged between 85.6% ± 8.2% and 93.6% ± 2.8%; for arachidonic acid it was 90% ± 5.8%. PE was the only PL whose fatty acid profile was slightly modified, showing a significant 15% decrease in arachidonic acid content (26.3% ± 3.7% in treated rats vs 30.9% ± 3.0% in controls). No significant change occurred in the fatty acid profile of other PL in the renal cortex.

Fatty acid analysis of the renal cortical neutral lipid subclasses. Table 5 shows the distribution pattern of fatty acids in FFA, triglycerides (TG) and cholesterol esters classes. These data indicate that changes occurred in the FFA class, with significant increases occurring in oleic (18:1) and linoleic (18:2) acids. No change was seen in arachidonic acid content. The unsaturation index was increased (209.6 ± 16.4 in controls vs 227.2 ± 13.2 in treated rats; $P < 0.05$). The fatty acid profile did not change in TG or cholesterol esters classes.

Discussion

Celiptium induced renal toxicity and lipid peroxidation in proximal convoluted tubules of rats. The affinity of this drug for the kidney has previously been shown both by tissue autoradiographic studies, in which distribution of [^{14}C]-celiptium demonstrated drug accumulation in the kidney, lung and liver [41], and by autoradiographic studies performed after microdissection of rabbit nephron, in which proximal intratubular localization was observed at 3 and 24 h after intravenous administration of [^{3}H]-celiptium (personal communication). Celiptium has induced dose-dependent renal toxicity in Wistar rats, and we reported tubular necrosis and tubulo-interstitial lesions [32]. In the present study we analysed the major changes in PL and neutral lipids of rat renal cortex after a single dose of 20 mg/kg celiptium.

By electron microscopy, a lipid overload was observed in proximal tubular cells. Histochemical techniques showed that these fatty deposits consisted of unsaturated hydrophobic lipids, and Holzcinger's method demonstrated that these lipids represented FFA deposits that were numerous and dispersed in proximal tubular cells. These observations corroborate the significant increases in FFA (×6) and glyceride levels (×1.5) observed in renal lipid extracts. The mass of TG increased slightly in parallel with a rise in FFA mass. A parallel decrease in total PL was demonstrated by lipid phosphorus assay, which particularly showed a decline in overall PE.

As the decrease in PE was accompanied by increases in FFA and TG, our study showed that the increase in FFA (day 2) preceded that in glycerides (day 4). Apart from oxidation, the main fate of exogenous fatty acids in the kidney involves esterification to triacylglycerol and glycerophospholipids: thus, we may assume that the increase in FFA levels is followed by TG synthesis in the renal cortex of treated rats. Indeed, if the fatty acid enzymatic pathway in the kidney is similar to that established for the liver, the kidney is not an important site of fatty acid synthesis [25, 45]. Moreover, it has been shown that increases in median fatty acid concentrations in cortical tubule suspension lead to increasing triacylglycerol levels and that gluconeogenic substrates stimulate TG synthesis [44]. In contrast, triacylglycerol decreased in the absence of exogenous fatty acids [46]. As other metabolic states such as starvation or diabetes mellitus can induce an increase in the triacylglycerol content of the rat kidney [11, 19], we ensured that the animals in our study were not diabetic (results not shown). In spite of the increase in triacylglycerol and its role in fatty acid stores for PL synthesis [44], we observed a decline in total PL after celiptium treatment. As both the rise in FFA and the decline in PE occurred on day 2, we hypothesized that the increase in FFA might be linked to a loss of fatty acids consequent to PL breakdown.

Previous findings of Auclair et al. [2] showed that N2-methyl-9-hydroxy-ellipticinium (NMHE) and its derivatives undergo spontaneous oxidation in aqueous solution, with molecular oxygen serving as the electron acceptor. Indeed, all ellipticine drugs that have a *p*-hydroxyl group in position 9 exhibit the ability either to transfer one electron to molecular oxygen to generate anion superoxide $O_2^{\cdot -}$ or to be oxidized by a peroxidase in the presence of hydrogen peroxide. With NMHE, the intermediate product is the quinone-imine N_2-methyl-9-oxoellipticinium (NMOE), which is highly electrophilic. Such intermediates may occur in vivo; a similar metabolism occurs in the liver and kidney [22, 26]. As kidney cells can induce biooxidative activation of celiptium in highly electrophilic intermediates [24], reactive species may be derived from celiptium metabolism in the kidney. Oxygen radicals or reactive species are known to participate in the lipid-peroxidation reaction process. Lipid peroxidation has been well studied and is known to be responsible for numerous effects in biological systems and for diseases such as drug-induced toxicity [9, 18, 37].

We therefore studied renal cortex lipids after celiptium treatment using lipid peroxidation as an end point. Celiptium treatment induced a 7- to 9-fold rise in lipid peroxides and a 1.7-fold increase in TBA reactivity. Although we know that most TBA reactivity is derived from the breakdown of lipid peroxides to MDA or aldehydes during the acid-heating stage of test conditions [10], this lesser increase may be attributed to the fact that these breakdown products may cross-link and aggregate amino groups of membrane proteins. Thus, the analysis of TBA-reactive substances is semi-quantitative and reflects only the presence of lipid peroxidation.

A decrease in polyunsaturated fatty acids was observed in total PL, along with a predominant loss of arachidonic acid. The fatty acid analysis of major PL shows changes in only the PE class, with a lower level of arachidonic acid occurring as the overall mass of PE declined. Indeed, the PE in renal cortex is particularly rich in polyunsaturated fatty acids, leading to greater susceptibility to peroxidative damage [39, 40]. Thus, the loss of polyunsaturated fatty acids, along with the presence of lipid peroxides and TBA-positive material, are indicators that a lipid-peroxidation process might occur in rat kidney treated with celiptium.

More particularly, our study indicated a decrease in arachidonic acid in renal PL, which paralleled an increase in lipid peroxides (×9) and FFA (×6). The GLC analysis of FFA detected oleate and linoleate as the main intact fatty

acids in neutral lipids. It is important to note that studies conducted in vitro with linoleic acid (18 : 3) under TBA test conditions showed no TBA-reactive material: in the absence of lipid peroxides, FFA do not undergo significant lipid peroxidation [10].

It has been shown that peroxidized lipids become preferential substrates for endogenous phospholipases [43]. The fatty acids that predominate in the sn-2 position of the glycerophospholipids comprise 78% of unsaturated fatty acids, whereas 42% are found in the sn-1 position [28]. Therefore, phospholipase A_2, known to act in the sn-2 position, is required for the release of these fatty acids and thus plays a protective role in membranes altered by lipid-peroxidation damage [14, 36, 42]. Using liposomes as model membranes, Sevanian and Kim [35] showed that phospholipase A_2 preferentially hydrolysed peroxidized fatty acid esters in PL membranes, whereas hydrolysis of about 15% of the intact and non-peroxidized fatty acids occurred simultaneously. Thus, in the present study, the action of phospholipases might explain the presence of both intact non-esterified fatty acids and peroxidized fatty acids in celiptium-treated renal cortex when a high overall decline in PE occurs.

It has recently been shown that lipid peroxidation might be an initial event in experimental acute renal failure such as ischemia or sodium maleate intoxication [15]. In contrast to these two models, in which no TBA-reactive substances accumulate in ischemic renal epithelia and in which lesions following maleate treatment more specifically implicate mitochondria, acute renal failure due to celiptium treatment is characterized by an increase in two breakdown products: (i) nonesterified fatty acids that are known to be toxic via detergent action on biological membranes or via alterations of the cytoskeleton and of contractile proteins in lymphocytes [13] and (ii) aldehydes that are known to be cytotoxic via a wide variety of inhibitory effects on cell functions and that are involved in protein damage via their high reactivity with proteins [8].

In conclusion, celiptium is an antitumor agent that is nephrotoxic in man and animals. Tubular proximal cells are the ultimate site of lesions, which subsequently lead to necrosis. In regard to the early accumulation of TBA-reactive substances (on day 2) followed by renal disfunction (on day 4), we suppose that cell necrosis did not participate in the lipid-peroxidation process. However, further studies, currently under way to determine which membranes are altered in this model injury, might enable us to better our knowledge of peroxidative injury in the rat kidney.

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