Biotransformation, transport and toxicity studies in rat renal proximal tubular cells

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STELLINGEN

1. Confluente monolayers van renale proximale tubuluscellen van Wistar ratten hebben geen actieve basolaterale transportsystemen voor glutathion conjugaten.
(Dit proefschrift)

2. De *in vitro* toxiciteit van glutathion geconjugeerde hydrochinonen is voornamelijk het gevolg van extracellulaire redox cycling.
(Dit proefschrift)

3. Metabolisme van thioether conjugaten van hydrochinonen tot gecycliseerde producten, zoals gemeten in een *in vitro* systeem, vindt ook plaats in *in vivo* systemen.
(Dit proefschrift)
(Rivera et al., 1994; Drug Metab. Dispos. 22, 503-509)

4. Het enzym γ-glutamyltranspeptidase heeft een dualistische functie: enerzijds initiëert het de detoxificatie van glutathion geconjugeerde hydrochinonen en anderzijds metaboliseert het glutathion geconjugeerde hydrochinonen tot meer toxische metabolieten.
(Dit proefschrift)
(Monks et al., 1995, Drug Metab. Rev. 27, 93-106)

5. Cryopreservatie van nierslices heeft waarschijnlijk geen effect op de γ-glutamyltranspeptidase en dipeptidase gemediëerde omzetting van glutathion conjugaten.
(Dit proefschrift)

6. De hypothese dat corticale neuronen tijdens het normale verouderingsproces niet afsterven maar gepreserveerd worden is vooralsnog onvoldoende aangetoond.
(Wickelgren, 1996, Science 273, 48-50)

7. Bij de herintroductie van verdwenen diersoorten, zoals de zeearend, spelen bijna uitsluitend ecologische argumenten een rol.
(Volkskrant, 7 augustus 1996)

8. De norm voor concentraties van zware metalen in water, zoals gehanteerd door het ministerie van VROM, houdt geen rekening met het fysiologisch welbevinden van vissen.

9. In tegenstelling tot de maatschappelijke verwachting is het niet mogelijk om de toxiciteit van nieuwe stoffen op een adequate wijze te evalueren met enkel proefdiervrij onderzoek.

10. Uit wetenschappelijk en beleidsmatig oogpunt valt een scheiding tussen het risicoschattings -en beheersingsaspect van de toxicologische beoordeling ten behoeve van de vaststelling van internationale residu normen voor diergeneesmiddelen toe te juichen. Of de internationale harmonisatie en de volksgezondheid hierbij gebaat zijn, valt te betwijfelen.

11. Het publiceren van een reeds ingevulde personeelsadvertentie is een immorele actie.

12. De opvatting dat er altijd wel kleine foutjes in het te drukken proefschrift zullen zitten heeft een geruststellende uitwerking op de promovendus.

13. Volgens berekeningen hoeft men voor een effectieve bestrijding van het fileprobleem bij het carpoolen niet eens met zijn tweeën in een auto te zitten.

14. Met de zogenaamde "sudden-death" maatregel is tevergeefs getracht het voetbal nieuw leven in te blazen.

Stellingen behorende bij het proefschrift:
" Biotransformation, transport and toxicity studies in rat renal proximal tubular cells "
Bert Haenen, Wageningen, 2 oktober 1996.

CONTENTS

Ter nagedachtenis aan mijn moeder

voor mijn vader

voor Sylvia en Youri

CHAPTER 1

GENERAL INTRODUCTION

General outline of the present thesis

The most important function of the kidney is to regulate fluid and electrolyte balance in the body within narrow limits. To that end, kidneys continuously filter blood in order to excrete metabolic waste products. In addition, filtered nutrients, salts and water are reabsorbed by the kidney in appropriate portions.

The unusual vulnerability of the mammalian kidney to the adverse effects of xenobiotics can be ascribed in large measure to its small mass in relation to its high blood flow (25% of the resting cardiac output) which results in large quantities of xenobiotics and their metabolites delivered to the kidneys. In addition, its high metabolic activity and its energy dependent reabsorptive functions are targets for chemical induced injury. Another important aspect in the kidneys' susceptibility to xenobiotics is the fact that, as a result of water reabsorption, kidney cells are exposed to increased concentrations of xenobiotics, an effect which may enhance toxicity.

According to the Proceedings of the Third International Symposium on Nephrotoxicity, more than 70% of the identified nephrotoxic compounds affect the proximal tubule. *In vivo*, proximal tubular cells can be exposed luminally (apically) to glomerulary filtrated and serosally (basolaterally) to non-filtrated xenobiotics. In addition, renal proximal tubular (RPT) cells contain basolateral transport systems to excrete waste products into the urine. However, the majority of the models developed so far to study proximal tubular toxicity used RPT cells either in suspension or cultured on solid supports. Since in these models basolateral transport systems are no longer or only partially active, less attention has been paid to the role of these transport systems in renal toxicity. Therefore, when studying mechanistic effects of xenobiotics *in vitro* one should use a model that mimics the *in vivo* situation as closely as possible in the sense that cells can be exposed both through the apical and basolateral route.

An important step ahead was made by Mertens *et al.* (1988) and Boogaard *et al.* (1990a) who studied apical and basolateral toxicity of nephrotoxicants on, respectively, $LLCPK_1$ and RPT cell monolayers cultured on porous supports of tissue culture inserts. Due to the absence of an important transport mechanism in $LLCPK_1$ cells, the present study has been extended towards the

use of primary cultures of RPT cells. The aim of the present study was to further refine the approach and to investigate the role of transport and biotransformation in the differential proximal tubular toxicity of nephrotoxic compounds, using a series of model compounds.

Physiology of the mammalian kidney

Biochemical heterogeneity and site-specific tubular injury

The majority of nephrotoxicants have their primary effects on discrete segments or regions of the kidney. The reasons underlying this site-specific injury in the kidney are complex but appear to be due in part to segmental differences in morphology, physiology and biochemistry.

In mammals, kidneys are paired, bean-shaped organs that have a complex architecture composed of at least 15 different cell types (Hewitt *et al.*, 1991). The smallest functional subunit is the nephron (Fig. 1). Depending on the species, the kidney contains 10^4 to 10^6 nephrons. There are several types of nephrons, but all of them consist of five main parts: the glomerulus, the proximal tubule, Henle's loop, the distal tubule and the collecting tubule.

At the glomerulus, arterial blood is filtered into the proximal tubule. The endothelial surface of the glomerular capillary forms a selective macromolecular filter with a cut off of approximately 75-100 Å. As a result, the ultrafiltrate is almost identical to deproteinized plasma. The presence of negatively charged glycoproteins on the surface of the glomerular capillary wall favors the filtration of neutral and positively charged solutes (Maddox and Brenner, 1991). As a result of a high blood flow, large amounts of xenobiotics are delivered to the glomerulus which is the first part of the nephron to come into contact with the xenobiotic. Apart from selective disposition of antibody complexes to glomerular basement membranes, which results from the interaction between negatively charged glycoproteins and cationic antigen-antibody complexes, glomerular damage due to exposure to xenobiotics is often non-specific (Madaio *et al.*, 1984).

After glomerular filtration of urine, the resulting ultrafiltrate is processed along the nephron and as a result, its composition changes. To that end, nephrons are supplied with an intricate network of capillaries enabling a tight contact between the bloodstream and the epithelial cells lining the tubules. Proximal tubules excrete metabolic waste products from the blood circulation into the ultrafiltrate. Proximal tubules reabsorb 50 to 60% of the glomerular filtrate. Essential nutrients (i.e. sugars, amino acids, proteins), cations (Na^+, K^+, Mg^{2+} and Ca^{2+}), anions (Cl^-, HCO_3^-, and PO_4^{3-}) and water are delivered back to the systemic circulation. Due to water reabsorption, the concentration of compounds, including xenobiotics, in the primary urine exceeds its concentration in the plasma. As a result, proximal tubular cells may be exposed to high concentrations of xenobiotics. Xenobiotics may also be sequestered in the proximal tubular

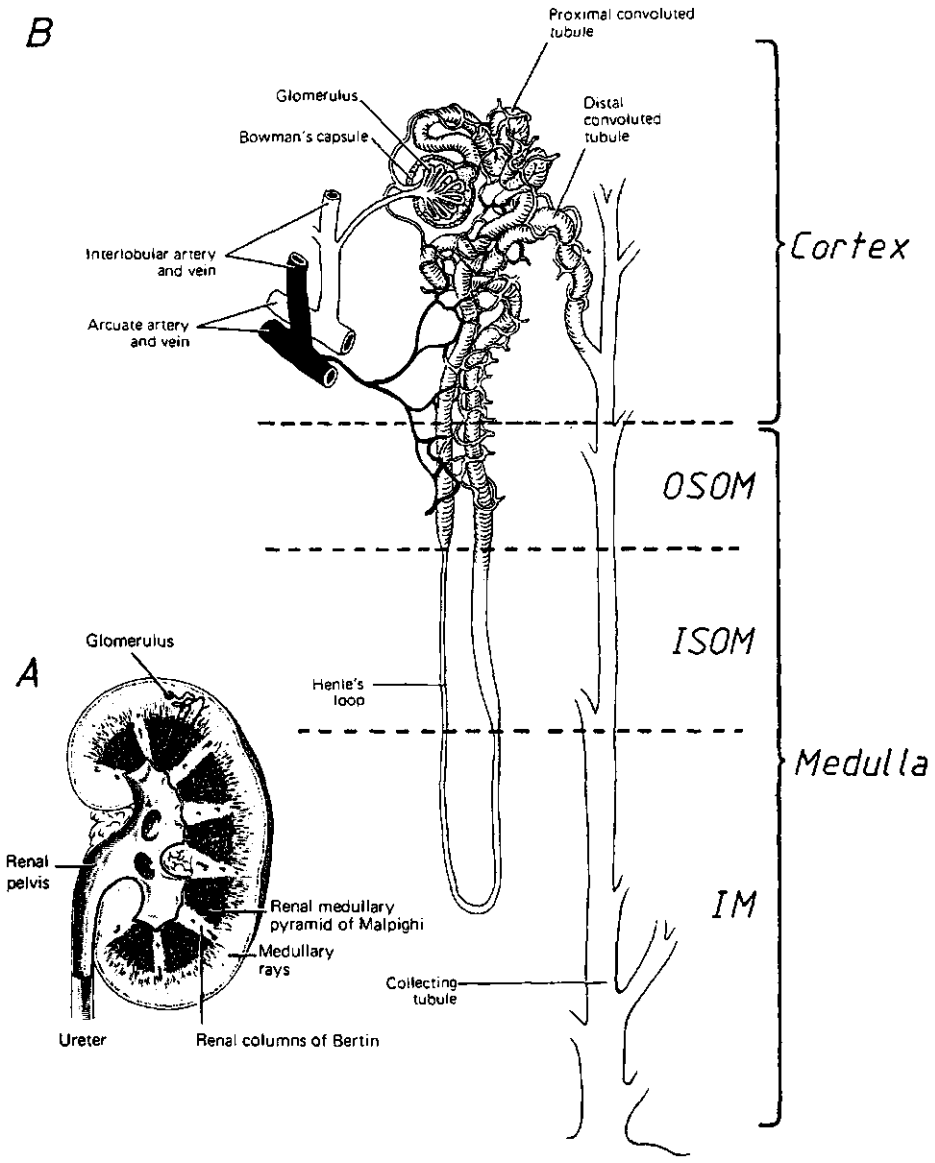


Figure 1. *Structural organisation of the kidney (A) and a nephron (B). Abbreviations: OSOM, outer stripe of the outer medulla; ISOM, inner stripe of the outer medulla; IM, inner medulla. (Adapted from Junqueira et al., 1977).*

cell at elevated concentrations compared to plasma by selective uptake mechanisms involved in tubular reabsorption.

Henle's loop extracts approximately 25% of filtered Na^+ and K^+ and reabsorbs 15-20% of water. Whereas the thin limb of Henle's loop acts primarily as a simple diffusion barrier, the thick ascending limb, which is impermeable to water, is involved in Na^+-K^+-Cl^- cotransport. The function of Henle's loop might be vulnerable to ischaemic injury. This results from the fact that Henle's loop is located in the medulla which is relatively hypoxic due to the fact that it receives only 10% of total renal blood flow.

The distal tubule and the collecting tubule also play a role both in water and Na^+ reabsorption. In addition, the distal tubule is responsible for the "fine tuning" of renal water and solute excretion. It regulates the pH of the urine and, depending on the need, K^+ is either secreted or reabsorbed. In general, the distal tubule appears not to be selectively damaged by most toxicants. However, amphotericin B impairs urine acidification and, as such, it primarily affects the distal tubule (Kinter and Short, 1993). According to some unknown mechanism, chronic abuse of analgesics, such as phenacetin, primarily affects medullary segments (Duggin, 1993). In addition, the general anesthetic methoxyflurane predominantly affects medullary segments in part by interfering with the action of vasopressin in Henle's loop and collecting tubule (Goldstein, 1993).

Thus, the highly differentiated functions in combination to its orientation in the nephron, predispose the proximal tubule to be the most vulnerable for toxicants of all nephron segments. The mammalian proximal tubule is not homogenous in structure. Therefore, in several animals, including the rat, rabbit, mouse and rhesus monkey, three morphologically distinct segments (S_1, S_2 and S_3) can be distinguished to comprise the convoluted part (pars convoluta) and straight part (pars recta) of the proximal tubule. The S_1 segment comprises the initial and middle portion of the proximal convoluted tubule. The S_2 segment extends from the rest of the convoluted segment to the initial portion of the straight segment. The S_3 segment comprises the distal (remaining) portion of the straight part of the proximal tubule. The specific transport characteristics of each segment determines in large part the site of xenobiotic-induced proximal tubular injury. Since the S_2 segment is predominantly involved in basolateral organic anion transport, this segment is particularly vulnerable to toxic xenobiotics present as organic anions. For similar reasons, aminoglycosides produce lesions in S_1 and S_2 segments and hexachlorobutadiene (HCBD) induces well-defined lesions in the S_3 segment (Walker and Duggin, 1988).

Biotransformation of glutathione conjugates: role in nephrotoxicity

Another important factor that determines the vulnerability of different nephron segments to xenobiotics is their distribution of biotransformation enzymes. Most phase I enzymes exhibit their highest activity in the renal cortex. The greatest activity of cytochrome P-450 is localised within

the proximal tubule. Compared to the liver, renal phase I metabolism is generally of minor importance (Inoue, 1985). However, the role of renal phase II enzymes is often more substantial. For example, glucuronidation of *p*-nitrophenol by renal uridinediphosphate (UDP) glucuronyltransferases probably accounts for approximately 20% of total excreted glucuronide in the rat (Diamond and Quebbeman, 1979). Renal sulfation seems to be quantitatively less important than glucuronidation (Diamond and Quebbeman, 1981).

Within the kidney, proximal tubules are especially equipped with enzymes that metabolise glutathione and glutathione-*S*-conjugates. γGlutamyltranspeptidase (γGT), dipeptidases and cysteine conjugate *N*-acetyltransferase metabolise glutathione conjugates into their corresponding mercapturic acids (Fig. 2) (Inoue, 1985). As a result, conjugation of potentially electrophilic compounds with glutathione (GSH) can lead to detoxification of the xenobiotic (Boyland and Chasseaud, 1969; Boyland, 1979) but, depending on the compounds, may also lead to activation into cytotoxic, mutagenic and carcinogenic compounds (Elfarra and Anders, 1984; Anders *et al.*, 1987; Monks and Lau, 1992, 1994).

GSH conjugation occurs spontaneously or can be catalyzed by a family of cytosolic, microsomal or mitochondrial glutathione *S*-transferases. Although these enzymes are identified in a wide variety of tissues and species, the liver is one of the most prominent organs for enzymatic GSH conjugation (Rushmore *et al.*, 1994).

Depending on the size of the molecule and the rate of glutathione conjugation, the resulting GSH *S*-conjugate can be excreted in the blood circulation or in the bile (Wahlländer and Sies, 1979). At a low rate of synthesis, excretion in the bile is the predominant route. GSH *S*-conjugates can be degraded by γGT and dipeptidases present in the biliary tree and in the luminal membrane of the intestine (Fig. 3) (Grafstrom *et al.*, 1979; Kozak and Tate, 1982). After cleavage of the γglutamyl group of the GSH *S*-conjugate by γGT and subsequent removal of the glycine moiety by dipeptidases, a cysteine *S*- conjugate is formed (Jones *et al.*, 1979; Okajima *et al.*, 1981). This conjugate can be transported back to the liver via the portal blood (Gietl *et al.*, 1991) and is ultimately delivered to the kidney as cysteine *S*-conjugate or as its corresponding mercapturic acid after intestinal or hepatic *N*-acetylation (Inoue *et al.*, 1984; 1987). As a result of this enterohepatic cooperation, different thioethers: glutathione, cysteine and *N*-acetylcysteine conjugates may reach the kidney.

Fate of thioether conjugates in the kidney

The kidney is the primary organ for clearance of circulating GSH and GSH *S*-conjugates (McIntyre and Curthoys, 1980). Although approximately 80% of plasma GSH is cleared by the kidneys (Haberle *et al.*, 1979), only 25% is removed via glomerular filtration. As a result, a significant amount of circulating GSH, and probably also GSH *S*-conjugates, has to be cleared

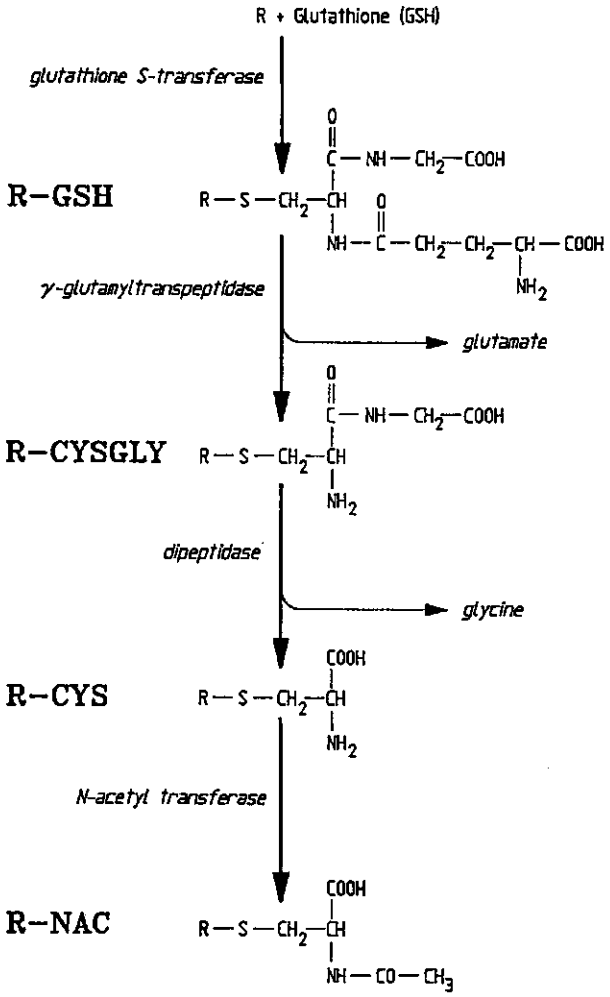


Figure 2. The mercapturic acid pathway.

via non-filtrating mechanisms involving proximal tubular uptake of GSH across the basolateral membrane (Lash and Jones, 1984) (Fig. 4). This indicates that basolateral exposure of renal proximal tubular cells to GSH *S*-conjugates contributes to a larger extent to the overall proximal tubular toxicity than apical exposure.

In support of this assumption, it was concluded by Lash and Jones (1984, 1985) that both GSH and GSH *S*-conjugates of trichloroethylene (DCVG: 1,2-dichlorovinylglutathione) are basolaterally taken up via a Na^+-coupled GSH transporter which was inhibited by probenecid. Probenecid is a selective and competitive inhibitor of organic anion transport (Mudge, 1980) without an effect on either synthesis of transport carriers or energy metabolism. The Na^+-coupled GSH transporter appeared to be specific for the γglutamyl moiety, since uptake of DCVG by basolateral membrane vesicles was only inhibited by GSH, GSSG and γglutamylglutamate and not by the corresponding cysteine conjugate: 1,2-DCVC.

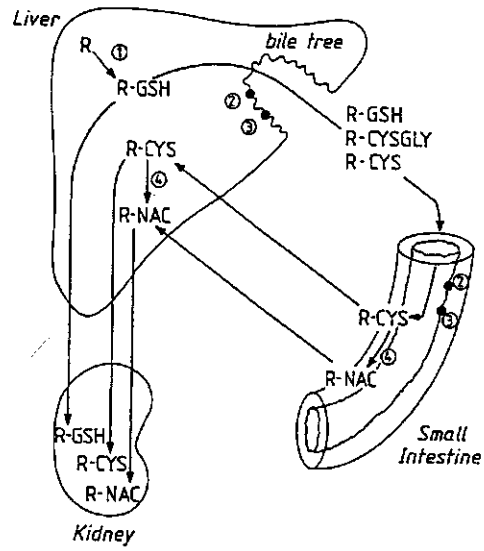


Figure 3. The role of enterohepatic cycling in the delivery to the kidney of different thioether conjugates. Enzymes involved are indicated by numbers: (1) glutathione S-transferase, (2) γglutamyltranspeptidase, (3) dipeptidase, (4) N-acetyltransferase. (Modified from Inoue, 1985).

GSH conjugates are not degraded intracellularly but are excreted into the tubular lumen by means of a specific carrier (Inoue and Morino, 1985). Until now, GSH excretion by means of an ATP-dependent glutathione *S*-conjugate export pump as described for hepatic canalicular plasma membranes (Inoue *et al.*, 1984) and erythrocytes (Kondo *et al.*, 1982) has not been detected in proximal tubular cells. In the tubular lumen GSH *S*-conjugates are enzymatically degraded by

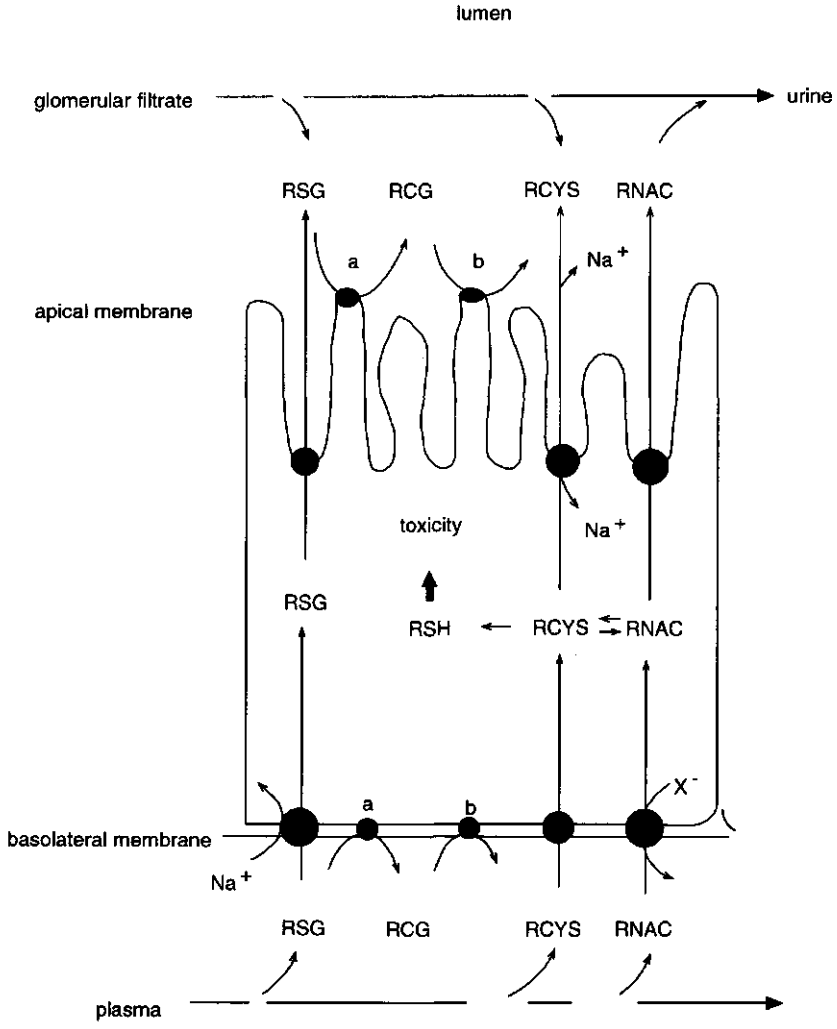


Figure 4. *Proximal tubular transport and metabolism of thioether conjugates. (a) γglutamyltranspeptidase, (b) dipeptidase. (RSG) glutathione conjugate, (RCG) cysteinylglycine conjugate, (RCYS) cysteine conjugate, (RNAC) N-acetylcysteine conjugate. (Adapted from Commandeur et al., 1995).*

γGT (Curthoys and Hughey, 1979) and dipeptidases like cysteinylglycine dipeptidase (Hughey *et al.*, 1978) and/or aminopeptidase M (Kozak and Tate, 1982) into the corresponding cysteine *S*-conjugates. The latter conjugates can be transported across the basolateral and apical membrane of the proximal tubular cell via both Na^+-dependent and independent transporters (Lash and Anders, 1989).

Na^+-dependent uptake of 1,2-DCVC in isolated rat renal proximal tubular cells was inhibited by probenecid and para-aminohippuric (PAH) acid indicating the involvement of a basolateral organic anion transporter (Lash and Anders, 1989). Further support for the involvement of such a transport mechanism came from experiments wherein 1,2-DCVC and other cysteine *S*-conjugates inhibited PAH transport in renal proximal tubules (Ullrich *et al.*, 1989).

Proximal tubular transport of cysteine *S*-conjugates also occurs via Na^+-dependent and -independent amino acid transport systems (Commandeur *et al.*, 1995; review). Since α-(methylamino)isobutyric acid (MeAIB), which is a specific substrate for system A, inhibits Na^+-dependent transport of 1,2-DCVC, it seems that this transport system, which is present in proximal tubular cells in both basolateral and brush border membrane (Murer and Gmaj, 1986), is responsible for the transport of this cysteine *S*-conjugate (Schaeffer and Stevens, 1987a, 1987b; Lash and Anders, 1989). The Na^+-dependent system L seems to play a role in the apical uptake of 1,2-DCVC in $LLCPK_1$ cells (Schaeffer and Stevens, 1987a) and in the basolateral transport of this conjugate in rabbit renal cortical slices (Wolfgang *et al.*, 1989). System T seems to be involved in the Na^+-independent basolateral uptake of the cysteine *S*-conjugate of HCBD (PCBD-CYS) in $LLCPK_1$ monolayers (Mertens *et al.*, 1990).

Since γGT and dipeptidases are also located in basolateral membranes of proximal tubular cells (Anderson *et al.*, 1980; Spater *et al.*, 1982; Abbott *et al.*, 1984), basolateral cysteine transporters do not only transport non-filtrated cysteine *S*-conjugates formed as a result of enterohepatic cycling of thioethers but also cysteine *S*-conjugates as a result of basolateral processing of glutathione *S*-conjugates. Cysteine *S*-conjugates are excreted in the lumen after intracellular *N*-acetylation into their corresponding *N*-acetylcysteine *S*-conjugates. Basolateral uptake of circulating *N*-acetylcysteine *S*-conjugates by renal proximal tubular cells occurs via a probenecid inhibitable and Na^+-dependent organic anion transporter (Lock *et al.*, 1986; Zhang and Stevens, 1989; Wolfgang *et al.*, 1989). It appears that the organic anion transporter is indirectly Na^+-dependent because this transporter is coupled to a Na^+-dependent transporter of dicarboxylates which exchanges dicarboxylates with organic anions (Ullrich and Rumrich, 1988). Microinfusion studies with isolated rabbit renal tubules showed that within the tubule, the activity of organic anion transport is highest in the S_2 segment (Woodhall *et al.*, 1978).

Mechanisms of glutathione conjugate-induced nephrotoxicity

Glutathione conjugates can be divided into three groups (Van Bladeren, 1988): direct-acting GSH conjugates, GSH conjugates in which the GSH moiety functions as a transporter of reversibly-bound electrophilic compounds and GSH conjugates which need further bioactivation via catabolism of the GSH and/or the xenobiotic derived moiety. Of this latter class of GSH conjugates only GSH conjugated hydroquinones will be discussed.

Direct acting GSH conjugates

Biotransformation of haloalkanes often involves both direct GSH conjugation and cytochrome P-450-mediated oxidative metabolism. Two observations support the consensus that direct GSH conjugation determines for most part the genotoxic potential of dihaloalkanes *in vivo*. Binding of 1,2-dibromoethane to DNA in hepatocytes was decreased by prior GSH depletion, but was not affected after inhibition of cytochrome P-450 (Sundheimer *et al.*, 1982). In addition, an increase in the occurrence of 1,2-dibromoethane-induced tumor formation in rats was observed after inhibition of oxidative metabolism (Wong *et al.*, 1982).

In addition to spontaneous conjugation of GSH to xenobiotics, this reaction is also catalysed by hepatic and, to a somewhat lesser extent, by renal glutathione *S*-transferases. After conjugation of GSH to 1,2-dichloroethane an *S*-(2-chloroethyl)glutathione conjugate is formed which, by intramolecular displacement of halogen and sulfur atoms, results in an alkylating episulfonium ion (Van Bladeren *et al.*, 1980). In view of the selective nephrotoxicity of 1,2-dichloroethane, it has been proposed that this selective toxicity is merely the result of a reactive metabolite formed in the liver of which the half life is apparently long enough to allow it to reach the kidney via the circulation (Elfarra *et al.*, 1985).

GSH conjugates as transporters of reactive electrophiles

Glutathione conjugation can also play a role in the delivery of electrophiles to distant nucleophilic sites of essential macromolecules (Baillie and Slatter, 1991). In this class of toxic GSH conjugates, the parent compound is in equilibrium with its GSH conjugate. As a result, the reactive compound is initially detoxified, but the release of the reactive moiety may occur distal to its site of formation. Thus, such GSH conjugates especially pose a risk to tissues where the (physico-chemical) conditions facilitate the release of the reactive compound. In this respect, γGT rich organs like the kidney may be at risk, since in a number of cases, the cysteine conjugate is much more labile than the parent glutathione conjugate (Monks and Lau, 1994).

GSH can also play a role in the delivery of heavy metals to the kidney. Both inorganic and methylmercury are transported to the kidney as complexes with GSH (Naganuma *et al.*, 1988;

Tanaka *et al.*, 1990). Indicative for a role of γGT in the renal accumulation of methyl mercury is the fact that mice treated with acivicin, a compound which irreversibly inhibits γGT (Lau *et al.*, 1988b), showed less renal accumulation of methyl mercury and an increased urinary excretion of methyl mercury than control-treated mice (Naganuma *et al.*, 1988). In addition, Tanaka and co-workers (1992) observed renal uptake of methyl mercury via the basolateral inorganic anion transport system.

Another class of toxic compounds known to be transported as glutathione adducts are the α,β-unsaturated carbonyls. These compounds reversibly conjugate with thiols via a Michael type reaction (Esterbauer *et al.*, 1975). Thiol adducts of several α,β-unsaturated aldehydes are cytotoxic. Cysteine conjugates of crotonaldehyde and *trans*-4-hydroxypentenal are carcinostatic, presumably due to the release of the alkylating aldehyde-moiety (Tillian *et al.*, 1976, 1978). The GSH conjugate of acrolein, *S*-(3-oxopropyl)GSH, appeared to be nephrotoxic in male Sprague-Dawley rats, an effect which could be prevented by acivicin (Horvath *et al.*, 1992). It was shown by Hashmi *et al.* (1992) that *S*-(3-oxopropyl)-*N*-acetylcysteine was toxic in $LLCPK_1$ cells and in rat renal proximal tubular cells by means of a novel bioactivation meachanism involving sulfoxidation of *S*-(3-oxopropyl)-*N*-acetylcysteine and subsequent elimination of acrolein.

The α,β-unsaturated ketone ethacrynic acid (EA) and its glutathione conjugate strongly inhibit glutathione *S*-transferase (GST) isoenzymes by transferring the ethacrynic acid-moiety to a thiol group of GST (Mannervik *et al.*, 1988; Ploemen *et al.*, 1990). EA is also a well-known diuretic. It inhibits renal salt reabsorption by inhibiting the uptake of Cl^- in the thick ascending limb of Henle (Koechel and Cafruny, 1975; Koechel, 1981). It has been shown that EA reaches this site of action after active proximal tubular secretion via a probenecid-inhibitable transport system (Beyer *et al.*, 1965). It seems that EA and EA-L-cysteine, both act on different sites of the Na^+-K^+-$2Cl^-$ -cotransporter. EA inhibits cotransport by alkylating -SH residues whereas EA-L-cysteine affects cotransport due to some stereospecific effect which is a property of the entire molecule (Palfrey and Leung, 1993).

GSH conjugates as precursors of toxic metabolites

The conjugation of quinones with GSH results in the formation of potent nephrotoxicants. The nephrotoxicity of bromobenzene and 1,4-benzoquinone in rats is probably mediated via its metabolism to 2-bromo-(di-glutathione-*S*-yl)hydroquinone (2-Br(diGSyl)HQ) and 2,3,5-(tri-glutathion-*S*-yl)hydroquinone (2,3,5-(triGSyl)HQ) respectively (Monks *et al.*, 1985, 1988b; Lau *et al.*, 1988a). Administration of only 10-20 μmol/kg of these GSH conjugates to rats is sufficient to cause enzymuria, glucosuria, elevations in blood urea nitrogen (BUN) and renal proximal tubular cell necrosis. The identification of 2,3,5-(triGSyl)HQ as an *in vivo* metabolite of hydroquinone (Hill *et al.*, 1993), which induces renal adenomas in male rats (Shibata *et al.*,

1991), points to a role of this GSH conjugate in hydroquinone-mediated nephrocarcinogenicity. Similarly, several glutathione conjugates of 2-*tert*-butylhydroquinone (TBHQ) have been implicated in the (nephro)carcinogenic potency of the phenolic food antioxidant 3-*tert*-butyl-4-hydroxyanisole (BHA) (Tsuda *et al.*, 1984; Lau *et al.*, 1994; Peters *et al.*, 1996).

The toxicity of GSH conjugates of hydroquinones is thought to result from oxidation of the hydroquinone to an alkylating quinone moiety which subsequently may covalently bind to non-sulfur nucleophiles and protein thiols (Monks and Lau, 1992). A direct correlation exists between the extent of covalent binding of 2-Br-[^{14}C]-HQ to renal macromolecules and elevations in BUN levels of rats treated with 2-Br-[^{14}C]-HQ (Lau and Monks, 1990). Another mechanism involved in the toxicity of quinone-thioether conjugates constitutes redox-cycling or autoxidation of (hydro)quinones which may lead to the production of reactive oxygen species (Wefers and Sies, 1983). In view of the nephrocarcinogenic potency of hydroquinones it is interesting to note that hydroxyl radicals seem to play a role in 2-Br-(di-glutathion-*S*-yl)hydroquinone induced cytotoxicity (Mertens *et al.*, 19993) and in hydroquinone-induced DNA damage (Leanderson and Tagesson, 1992).

γGT dependent toxicity of GSH conjugated hydroquinones

The tissue selectivity of 2-Br-(diGSyl)HQ and 2,3,5-(triGSyl)HQ appears to be the result of their targetting to renal proximal tubular cells by brush border γGT (Monks *et al.*, 1985; Lau *et al.*, 1988a). The subsequent action of γGT and dipeptidases converts the GSH conjugate into a cysteine conjugate which can be taken up by the proximal tubular cell. Inhibition of renal γGT by acivicin protected rats against 2-Br-(diGSyl)HQ and 2,3,5-(triGSyl)HQ mediated renal toxicity. As a result of acivicin treatment, the uptake of presumably 2-Br-(di-cystein-*S*-yl)HQ in tubular epithelial cells is inhibited (Lau *et al.*, 1988b) and the urinary excretion of the parent GSH-conjugate is increased (Lau and Monks, 1990). Since 2-Br-(di-cystein-*S*-yl)HQ and 2,3,5-(tri-cystein-*S*-yl)HQ are more readily oxidized to their corresponding quinones than the analogous GSH conjugates (Monks *et al.*, 1994; Hill *et al.*, 1994), γGT serves to deliver a more readily oxidizable and thus more reactive metabolite to renal epithelial cells.

γGT dependent detoxification of GSH conjugated hydroquinones

In contrast to the results obtained with 2-Br-(diGSyl)HQ and 2,3,5-(triGSyl)HQ were the observations that acivicin potentiated 2,5-dichloro-3-(glutathione-*S*-yl)hydroquinone and 2,5,6-trichloro-3-(glutathion-*S*-yl)hydroquinone induced nephrotoxicity (Mertens *et al.*, 1991). In other words, acivicin prevented γGT-mediated detoxification of these GSH conjugates and thus prevented the cellular uptake of the corresponding cysteine-conjugate. The observed effect is suggestive for an extracellular mode of toxicity. To explain this effect, it has been hypothesized

by Monks (1995) that acivicin prevents the formation of an 1,4-benzothiazine product via cyclization of the cysteinylglycine and/or cysteine conjugate (Fig. 5). Since this reaction eliminates the reactive quinone function from the molecule, it can be considered as a detoxification reaction (Monks *et al.*, 1990). The presence of the *N*-acetyl group in the mercapturate prohibits condensation of the cysteine amino group with the quinone carbonyl group. As a result, *N*-acetyl cysteine conjugates cannot undergo the cyclization reaction and thus retain the ability to redox cycle. In agreement with this, only the *N*-acetyl-cysteine conjugate of menadione (2-methyl-1,4-naphtoquinone) and not the GSH conjugate was nephrotoxic both *in vitro* (Brown *et al.*, 1991) and *in vivo* (Lau *et al.*, 1990).

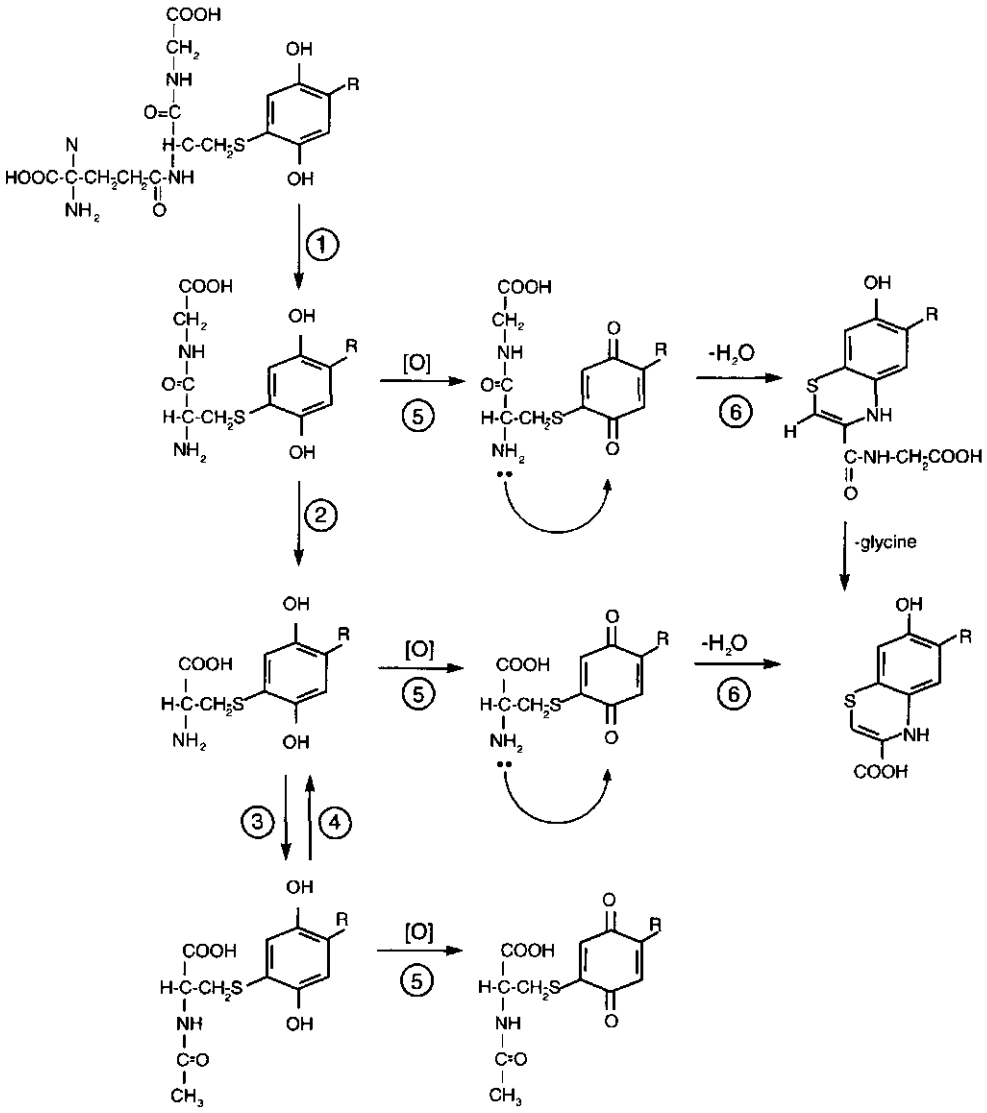
In conclusion, whether γGT catalyzes the activation or detoxification of quinol-GSH conjugates depends upon their relative ease to undergo oxidation, macromolecular arylation and intramolecular cyclization.

Evidence against the role of β-lyase in quinone-thioether mediated nephrotoxicity

The nephrotoxicity of thioether conjugated halogenated alkenes depends on their metabolism by cysteine conjugate β-lyase. As a result, thiol compounds are formed which may be very reactive themselves or which may rearrange to form highly reactive intermediates like thioketenes or thionoacylhalides (Dekant *et al.*, 1987; Commandeur *et al.*, 1989).

However, β-lyase does not appear to play a major role in quinol-GSH mediated nephrotoxicity. Pretreatment of rats with aminooxyacetic acid, an inhibitor of β-lyase, dit not protect animals from 2-Br-(diGSyl)HQ mediated nephrotoxicity (Monks *et al.*, 1988b). In addition, the renal toxicity of 6-bromo-2,5-dihydroxy-thiophenol, a putative β-lyase catalyzed metabolite of 2-Br-3-(GSyl)HQ, depended upon the quinone function rather than the thiol function (Monks *et al.*, 1988a). Both 2-Br-(di-cystein-*S*-yl)HQ and 2-Br-(di-*N*-acetylcystein-*S*-yl)HQ induced proximal tubular toxicity in rats (Monks *et al.*, 1991). β-lyase does not play a role in the development of toxicity when 2-Br-(di-cystein-*S*-yl)HQ is delivered to proximal

Figure 5. *Disposition of GSH-conjugated hydroquinones. After metabolism of the GSH-conjugated hydroquinone by γGT (1), a cysteinylglycine conjugate arises which after dipeptidase mediated cleavage (2) of the glycine-moiety results in a cysteine conjugate. The cysteine conjugate is either N-acetylated into the corresponding N-acetylcysteine conjugate (3) or deacetylated to yield the cysteine conjugate (4). However, data suggest that oxidation of cysteinylglycine and/or cysteine conjugated hydroquinones into their corresponding quinones is the major route of metabolism (5). Condensation of the cysteine amino group with the adjacent quinone carbonyl group constitutes an intramolecular cyclization and leads to 1,4-benzothiazine formation (6).*



tubular cells either via direct administration to rats or via γGT mediated metabolism of 2-Br-(diGSyl)HQ. However, when 2-Br-(di-cystein-*S*-yl)HQ is delivered to proximal tubular cells intracellularly via deacetylation of 2-Br-(di-*N*-acetylcystein-*S*-yl)HQ, β-lyase does play a role in the observed nephrotoxicity (Monks *et al.*, 1991). Since neither cysteine nor *N*-acetylcysteine

conjugates could be identified as major *in vivo* metabolites of 2-BrHQ (Lau and Monks, 1990), possibly due to oxidative cyclization of the corresponding cysteinglycine and/or cysteine conjugate, β-lyase seems to play a minor role in quinol-GSH conjugate induced nephrotoxicity.

Renal proximal tubular cells as in vitro models for studying nephrotoxicity

In the past decades various experimental models have been developed to study the mechanisms involved in chemical-induced nephrotoxicity. These models range from intact animals to *in vitro* models like the *in situ* perfused kidney, renal slices, suspensions of renal proximal tubules, renal proximal tubular cells either in suspension or in culture and various cell lines of proximal tubular origin. In using these models for studying chemical-induced nephrotoxicity, one has to be aware of their advantages and limitations. Therefore, it largely depends on the question to be answered which is the most appropriate system to be used.

In this section, the *in vitro* models will be discussed in terms of their usefullness for studying nephrotoxicity with special emphasis on the use of rat renal proximal tubular cells as primary cultures and rat renal cortical slices, since these *in vitro* systems have been used in this thesis.

The isolated perfused kidney

The isolated perfused kidney (IPK) is the only *in vitro* model in which chemical-induced nephrotoxicity can be assessed in the presence of an intact vascular supply and normal anatomical connections between the nephrons (reviewed in Maack, 1986). Since the IPK is an intact organ, it can reproduce the toxicity seen *in vitro* for very acute toxicants (Brezis *et al.*, 1984). The IPK can provide valuable data on renal transport (Hori *et al.*, 1988; Tanigawara *et al.*, 1990; Mihara *et al.*, 1993) and biotransformation (Schrenk *et al.*, 1988; Davison *et al.*, 1990; Hill *et al.*, 1994) of xenobiotics, but it still remains in many respects a black box. Interactions between several cell types makes it difficult to study the role of one particular cell type. Because renal biotransformation capacity of the IPK deteriorates rapidly, the IPK can only be used for about 4h. For this reason, the IPK is not an appropriate model to study the dose-relationship or the time-course of an effect of a chemical on a specific IPK function. In addition, perfusion pressure and perfusate composition has a major effect on the performance of the IPK (Brezis *et al.*, 1986). Since one animal is needed for each IPK preparation, this *in vitro* model does not contribute to a reduction in the use of laboratory animals.

Renal cortical slices

This tissue preparation has proven to be a succesfull *in vitro* system in assessing chemical-induced renal toxicity. Slices can be prepared from many areas of the kidney (Gandolfi and Brendel, 1990), however, slices from the cortical region have gained the greatest interest since this region is the most common target for renal toxicants (Wolfgang *et al.*, 1989a).

Slices are in particular suitable to study direct effects of chemicals without the confounding variables such as alterations in renal blood flow, renal anoxia and the contribution of metabolites originating from the liver. Similar to the IPK, renal cortical slices retain the multicellular composition and histological architecture of the tissue as well as intact membrane transporters and receptors. In contrast to the IPK, slices can be easily prepared from one kidney in significant amounts, thereby reducing the amount of laboratory animals needed.

The nephrotoxic potential of chemicals in renal cortical slices can be evaluated using parameters such as accumulation of *p*-aminohippuric acid and tetraethylammonium, intracellular K^+ levels and lactate dehydrogenase release (Smith *et al.*, 1981; Bach and Lock, 1982; Ruegg *et al.*, 1989; Trevisan *et al.*, 1992).

Slices can be used to evaluate *in vivo* effects when taken from animals sacrificed after exposure to a xenobiotic compound. In addition, the *in vitro* effects of chemicals can be tested by taking slices from untreated animals. In this respect, the *in vitro* nephrotoxicity of a number of renal toxicants in renal cortical slices, correlates well with their *in vivo* nephrotoxicity. In other words, renal cortical slices have proven to be a valuable tool for predicting *in vivo* effects of acute nephrotoxicants (Smith, 1988; Wolfgang *et al.* 1989b).

In the last decade, tissue slice preparation and culture conditions have been improved. This resulted in the preparation of ultrathin slices which can be maintained in culture for 72h under optimum oxygenation conditions (Ruegg *et al.*, 1987; Wright and Paine, 1992; Leeman *et al.*, 1995). In addition, these slices can be examined by light microscopy leading to a qualitative assessment of nephrotoxic injury of several cortical cell types in one slice simultaneously (Phelps *et al.*, 1987; Wolfgang *et al.*, 1989b). The improved culture conditions makes tissue slices, next to their role as screening tool for predicting (acute) toxicity, particularly valuable for studying mechanisms underlying toxicity at low concentrations of xenobiotics upon prolonged exposure.

Renal proximal tubules

Perfused isolated proximal tubules have proven to be a succesfull model to study electrophysiological and transport phenomena in this part of the kidney (for review, see Pritchard and Miller, 1993). Its laborious isolation and preparation yielding minute amounts of tubules (Barfuss *et al.*, 1979), precluded perfused renal tubules from in nephrotoxicity studies. In contrast, suspensions of renal tubules are widely used for evaluating effects of nephrotoxicants on

respiration, biotransformation and transport processes (Schäli and Roch-Ramel, 1981; Zhang and Stevens, 1989; Schnellman, 1989; Miller and Schnellmann, 1995). Tubules isolated via mechanical disruption generally exhibit better preserved cell membranes and transport function than collagenase-isolated renal tubules (Brendel *et al.*, 1993). In contrast, renal proximal tubules isolated with collagenase appear to be maintained in culture for longer periods than mechanically-derived systems (Gandolfi and Brendel, 1990). A disadvantage of tubules in suspension is the fact that the apical membrane may not always be completely accessible to the incubation medium due to a collapse of the tubule (Green *et al.*, 1989).

Renal proximal tubular cells in suspension

In general, isolated RPT cells can be obtained by preparative fractionation of the renal tissue. In this respect, two major procedures can be distinguished based either on mechanical disruption of the renal cortex (Toutain *et al.*, 1989) or on enzymatic digestion and subsequent purification of the cell suspension by isopycnic centrifugation (Boogaard *et al.*, 1989). Suspensions of isolated RPT cells have been succesfully used to study biochemical properties of these cells and their susceptability to xenobiotics (Lash and Tokarz, 1989; Boogaard *et al.*, 1990; Vamvakas *et al.*, 1992).

However, the limited life span of RPT cells in suspension constitutes a major drawback. Even under optimum conditions, viability of RPT cells in suspension declines after 3-4h of incubation (Boogaard *et al.*, 1989). Therefore, to elicit a rapid response, these cells may be exposed to artificially high concentrations of xenobiotics. Since artifacts can be induced by this protocol, the results obtained could be of limited relevance for the *in vivo* situation. In addition, the absence of cell-cell contacts and cellular polarity of cells in suspension constitutes another limition of this system. The apical and basolateral membranes of RPT cells, which are exclusively in contact with the primary urine and the blood, respectively, contain polarized transport systems. Since RPT cells in suspension lack this functional polarity, suspensions of RPT cells cannot be used for studying PT transport phenomena which are of paramount importance in renal handling and accumulation of xenobiotics. In contrast, RPT cells in culture exhibit cellular polarity which makes this system of particular interest to study RPT uptake of xenobiotics. The characteristics of cultured RPT cells as primary or as established cell lines will be described below.

Renal proximal tubular cells in culture

primary cell cultures

Primary cultures of RPT cells have been obtained from isolated proximal tubules or cells by microdissection and immunodissection techniques (Toutain *et al.*, 1992). Primary cultures have been initiated from both convoluted and straight proximal tubules after microdissection of rabbit and human renal slices (Wilson *et al.*, 1985, 1987). Because rat kidneys have smaller nephrons

and contain a large amount of connective tissue, microdissection of the rat kidney is apparently impossible without prior proteolytic treatment (Jung *et al.*, 1989). Although microdissection is the most effective method to obtain pure cultures of proximal tubule segments, the fact that this method yields only a limited number of cells explains why this method is not widely used in xenobiotic-induced RPT toxicity. The immunodissection technique, however, provides larger amounts of renal cells (Smith and Garcia-Perez, 1985). With this method, Stanton *et al.* (1986) cultured rat RPT cells by using a monoclonal antibody against microvillus membrane proteins of rat renal cortical cells. The relatively large amount of monoclonal antibody needed in this procedure prevented its application on a larger scale.

The most widely used technique to obtain primary cultures of RPT cells involves preparative fractionation of renal cortex. Primary cultures obtained in this way retain many of the biochemical properties characteristic for proximal tubules. For instance, confluent monolayers of proximal tubular cells in primary culture exhibit a typical cobblestone morphology and multicellular dome formation indicating the presence of transepithelial solute and water transport. In addition, RPT cell cultures exhibit brush-border membrane-associated γGT activity and Na^+-dependent transport of glucose and phosphate (Boogaard *et al.*, 1990; Toutain *et al.*, 1991). In addition, these cells exhibit probenecid-inhibitable PAH transport (Boogaard *et al.*, 1990) and parathyroid hormone-sensitive cAMP production (Toutain *et al.*, 1991).

In primary cultures, cellular differentiation and survival depends on the presence of various growth factors in the culture medium. Rabbit and human RPT cells form confluent monolayers and express differentiated functions when cultured in hormonally defined serum free medium (Detrisac *et al.*, 1984; Toutain *et al.*, 1991; Courjault-Gautier *et al.*, 1995). Rat and dog RPT cells never reached confluency and showed rapid dedifferentiation when cultured in these media (Rao *et al.*, 1989; Boogaard *et al.*, 1990). However, RPT cells isolated from rat, dog and pig kidneys can be successfully grown in serum-supplemented media (Stanton *et al.*, 1986; Rao *et al.*, 1989; Boogaard *et al.*, 1990; Kruidering *et al.*, 1993). Obviously, these RPT cells depend on unidentified growth factors present in (fetal) serum for optimal attachment and growth.

However, serum containing media might also stimulate growth of fibroblasts. This can largely be overcome by replacing L-valine and L-arginine in the medium by D-valine and L-ornithine, respectively, since fibroblasts cannot convert the former two amino acids (Leffert and Paul, 1973; Gilbert and Migeon, 1975).

As a consequence of their prolonged life span, cultured cells can be used to study the effects of xenobiotics at low concentrations relevant for the *in vivo* situation. The usefulness of RPT cell cultures in this context depends largely on the preservation of biotransformation capacity. During culture, the cytochrome P-450 activity in both rabbit and rat RPT cell cultures declines rapidly. In contrast, total glutathione content in RPT cell cultures remains stable. RPT cells in culture retain significant activities of enzymes playing a role in the mercapturic acid pathway like

glutathione *S*-transferase, γGT, dipeptidase, acylase and β-lyase essential for the expression of toxicity in the proximal tubule (Bruggeman *et al.*, 1989; Aleo *et al.*, 1990; Boogaard *et al.*, 1990b; Toutain *et al.*, 1991).

In addition to the culture medium, composition of the substratum also determines phenotype expression of cultured RPT cells. *In vivo*, renal tubular epithelia lie on a basement membrane of specific composition containing collagen type IV, laminin and fibronectin. Human kidney tubular cells maintained a highly differentiated state when cultured on a basement membrane substrate (Yang *et al.*, 1987). Rabbit proximal tubules purified by Percoll gradient centrifugation attach only on plastic coated with collagen. Rabbit RPT cells cultured in this way are more polarized and have abundant microvilli (Bello-Reuss and Weber, 1986).

The development of porous-bottom dishes for culture of polarized cells made it possible to study both vectorial transport of compounds and effects of xenobiotics on either the apical or basolateral surface of the cell. In addition, many epithelia including RPT cells differentiate more on porous surfaces than on plastic tissue culture dishes (Steele *et al.*, 1986; Courjault-Gautier, 1995). Therefore, RPT cells cultured on porous supports to confluent, polarized and differentiated monolayers seem to constitute an *in vitro* model closely resembling the *in vivo* sitation.

Cultured cell lines

Several cell lines of renal proximal tubular origin have been established (Gstraunthaler, 1990). Their rather quick disposal without laborious isolation and their virtual eternal life constitutes an advantage over primary cell cultures. One of the best characterized cell lines of proximal tubular origin is the $LLCPK_1$ cell line. $LLCPK_1$ cells closely resemble proximal tubular cells in many aspects and as a result, they have been of great importance in understanding renal physiology. Although continuous epithelial cell lines retain a number of differentiated properties of their ancestor cells (Gstraunthaler, 1988), cultured epithelia have lost some of their *in vivo* characteristics during adaptation to tissue culture. For instance, $LLCPK_1$ cells have lost functional basolateral organic anion transport (Rabito, 1986), which explains the lack of toxicity by mercapturic acids of halogenated alkenes (Stevens *et al.*, 1986; Mertens *et al.*, 1988) which are toxic *in vivo* (Lock and Ishmael, 1985). In addition, the $LLCPK_1$ cell line is not a homogeneous cell population.

Scope of the thesis

From the foregoing it is clear that transport systems play an essential role in nephrotoxicity. To

understand the nephrotoxicity of xenobiotics and their metabolites an *in vitro* model is required in which proximal tubular transport and metabolism of these compounds closely resembles the *in vivo* situation. Therefore, the main objective of this study was to develop such an *in vitro* system. We initially used confluent monolayers of proximal tubular cells cultured on porous supports of tissue culture inserts. Using this model, the apical and basolateral toxicity of thioether conjugates of two model (hydro)quinone compounds is studied (Chapters 2 and 3). Proximal tubular transport and metabolism of a glutathione conjugated nitrobenzene compound by this model was investigated in Chapter 4. Transport and metabolism of glutathione conjugated menadione is compared with the reversible conjugate of ethacrynic acid and glutathione (Chapter 5). Since several crucial metabolic and transport steps seem to disappear during culture, *N*-acetylation and the transport of organic acids, in Chapter 6 also freshly isolated and cryopreserved rat renal cortical slices were evaluated as an *in vitro* tool for renal metabolism.

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CHAPTER 2

Differential detoxification of two thioether conjugates of menadione in confluent monolayers of rat renal proximal tubular cells

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SUMMARY

This *in vitro* study describes proximal tubular toxicity of quinone thioethers after incubation of these compounds on either side of a renal proximal tubular (RPT) cell monolayer. These cells were cultured to confluency on porous supports of tissue culture inserts and the apically and basolaterally induced toxicity of two thioether conjugates of menadione (2-methyl-1,4-naphthoquinone) was investigated. As judged by lactate dehydrogenase (LDH)-leakage, the glutathione (GSH) conjugate of menadione (MGNQ) was only toxic after basolateral challenge. However, after inhibition of γglutamyltranspeptidase (γGT) by acivicin, MGNQ was also toxic after apical challenge. The mercapturic acid of menadione [M(NAC)NQ] displayed cytotoxicity both after apical and basolateral challenge. From the basolateral side, MGNQ and M(NAC)NQ-induced cytotoxicity could be enhanced by inhibition of the organic anion transport system with probenecid. Inhibition of β-lyase did not influence M(NAC)NQ-induced cytotoxicity. In addition, inhibition of intracellular *N*-deacetylation of M(NAC)NQ using paraoxon potentiated the observed toxic effect. Thus, it appears that the MGNQ and M(NAC)NQ induced cytotoxicity is the result of extracellular events, presumably redox cycling. Putative uptake of the conjugates is likely to be associated with detoxification of these compounds.

INTRODUCTION

Quinones constitute a group of electrophiles which are widely distributed in nature. Many quinones are present in the diet and many anti-cancer drugs contain the quinone nucleus (Ames, 1983; Monks *et al.*, 1992). In addition, quinones are formed from numerous xenobiotics via

biotransformation (Monks *et al.*, 1992; Den Besten *et al.*, 1991).

The toxicity of a large number of quinones stems from their ability to undergo "redox cycling" by which an oxidative stress is created (Smith *et al.*, 1985). In addition, many quinones are electrophilic and can react with cellular nucleophiles such as proteins and non-protein sulfhydryls.

Glutathione is the major cellular non-protein sulfhydryl (Reed and Meredith, 1985). Conjugation of xenobiotics like quinones with GSH has been associated with detoxification and excretion (Chasseaud, 1979). However, it has become clear that quinone-GSH conjugates can be very potent nephrotoxicants (Van Bladeren, 1988; Monks *et al.*, 1990b). As GSH conjugation of quinones does not destroy the quinone nucleus, it may be evident that the quinone-GSH conjugate is still able to redox cycle with the concomitant formation of reactive oxygen species (Wefers and Sies, 1983).

Generally, GSH *S*-conjugates are converted via the formation of cysteine *S*-conjugates to the corresponding mercapturates (*N*-acetyl-L-cystein *S*-conjugates). As these reactions also occur in liver and intestinal tissue, different metabolites of the mercapturic acid pathway may reach the kidney (Inoue, 1985). Of these quinol-*S*-conjugates, a substantial number has proven to be potent renal toxicants, especially for proximal tubular cells (Monks *et al.*, 1985; Mertens *et al.*, 1991; Monks *et al.*, 1991). In contrast to the pathway of haloalkene-mediated nephrotoxicity, metabolism by β-lyase does not appear to play a major role in the nephrotoxicity of quinol-*S*-conjugates (Monks *et al.*, 1988, 1991).

In vivo, proximal tubular cells can be exposed luminally (apically) to glomerulary filtrated and serosally (basolaterally) to non-filtrated quinol-conjugates. Therefore, when studying the effects of renal toxicants *in vitro* one should use a test system that mimics the *in vivo* situation as closely as possible, in order to augment the physiological significance of the effects found *in vitro*. In view of this, cultures of RPT cells on porous supports of tissue culture inserts are preferable to cell cultures on solid supports or cell suspensions. Because of the relative instability of benzoquinone-*S*-conjugates (Mertens *et al.*, 1990), we studied the toxicity of two thioether conjugates of menadione that remain relatively stable in the oxidized form (Brown *et al.*, 1991).

We report here for the first time the differential toxicity of two quinone thioethers, 2-methyl-3-(glutathion-*S*-yl)-1,4-naphthoquinone (MGNQ) and 2-methyl-3-(*N*-acetylcystein-*S*-yl)-1,4-naphthoquinone [M(NAC)NQ] to confluent cultures of RPT cells on porous supports of tissue culture inserts.

MATERIALS AND METHODS

Chemicals

Menadione (2-methyl-1,4-naphthoquinone) was obtained from Aldrich (Brussels, Belgium). GSH and *N*-acetyl-L-cysteine were from Janssen (Beerse, Belgium). Collagenase (from Clostridium histolyticum) was purchased from Boehringer (Mannheim, Germany). Nycodenz (iohexol) was from Nycomed AS (Oslo, Norway). AT-125 (acivicin), dextran Blue and probenecid were bought from Sigma (St. Louis, MO, USA). Amino-oxyacetic acid was from Merck (Munchen, Germany). Paraoxon was purchased from Riedel de Haën (Woerden, The Netherlands). All other reagents were of the highest grade commercially available.

Animals

For all experiments, female Wistar rats (Harlan CPB, Zeist, The Netherlands) weighing 180-230 g were used. The animals had free access to a commercial diet (RMH-B: Hope Farms, Woerden, The Netherlands) and tap water, and were kept on a 12/12h day/night cycle.

Cell isolation and cell culture

Proximal tubular cells were isolated following a procedure from Boogaard *et al.* (1989). RPT cells (2.10^5 cells/cm^2) were cultured on collagen coated transwell cell culture chambers (Costar, Badhoevedorp, The Netherlands) at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium used was William's E Medium (WE) (Flow, Irvine, Scotland) supplemented with 10% fetal calf serum (FCS; Gibco, Glasgow, Scotland) and gentamycin (50 μg/ml) (Gibco, Paisley, Scotland).

Monolayer confluency

As judged by light microscopy, monolayers reached confluency four days after plating. At that time, confluency was checked further by measuring the transepithelial electrical resistance (TEER) using a Millicell ERS Apparatus (Millipore, Bedford, MA). The measured TEER values were calibrated by assessing dextran blue (DB)-leakage into the culture chambers. To that end, a non-toxic DB concentration (1 mg/ml) was dissolved in Earl's Balanced Salt Solution (EBSS) and added to one compartment of the chamber. The appearance of DB in the opposite compartment was measured spectrofotometrically at λ 615 nm over 4h. DB-leakage was also assessed in the absence of cells to determine the barrier properties of the insert membrane. The sensitivity of the assay was such that 1% leakage could be detected. After TEER-value calibration, confluency was routinely checked by TEER determination only. For experiments, only monolayers displaying no DB-leakage were used.

Conjugate synthesis

MGNQ and M(NAC)NQ were synthesized as described by Brown *et al.* (1991) using a modified method originally described by Nickerson *et al.* (1963).

(MGNQ) Twenty mmoles of recrystallized menadione were dissolved in 210 ml of 95% ethanol. To this solution 20 mmoles of GSH dissolved in 70 ml water were added. After an overnight incubation in the dark at 4°C, brown crystals were collected by filtration, washed with 95% ethanol and dried at room temperature *in vacuo* over soda lime. To the dried crystals, water was added (50 ml/gram crystals) and the stirred mixture was heated and filtered. Two volumes of cold 95% ethanol were added to the filtrate, which was placed in the dark overnight at 4°C. The resulting clear crystals were harvested, washed with cold 95% ethanol and dried *in vacuo*. The molar percentage yield of the reaction was approximately 20%.

(MNACNQ) Twenty mmoles of recrystallized menadione were dissolved in 210 ml 95% ethanol. This solution was mixed with a solution of 20 mmoles of *N*-acetyl-L-cysteine in 70 ml water. The mixture was left overnight in the dark at 4°C, then 300 ml of water were added and left overnight again in the dark at 4°C. The crystals formed were harvested, washed with cold water and dried *in vacuo*. The molar percentage yield of the reaction was about 20%. Purity of the conjugates was checked by high performance liquid chromatography (HPLC) using a reversed-phase analytical column 150x4,6 mm; Lichrosorb 5RP18 (Chrompack, Middelburg, The Netherlands). Conjugates were eluted with a linear gradient of methanol/1% acetic acid in water (20:80 to 100:0) at a flow rate of 1 ml/min over 60 min. As judged from HPLC analysis, purity of the conjugates was at least 95%. Purity and identity of the conjugates was assessed further by field desorption mass spectral analysis using a MS 902 equipped with a VG-ZAB console. At emitter current 24-30 mA, a signal was observed at *m/e* 478 and 334 which correspond to MGNQ and M(NAC)NQ, respectively (Brown *et al.*, 1991).

Cytotoxicity assay

Confluent monolayers were washed with EBSS containing 10 mM $NaHCO_3$ and 20 mM HEPES (pH 7,4). Apical or basolateral treatment of the monolayers with the conjugates was performed for 4h at 37°C in a humidified atmosphere containing 5% CO_2. Both conjugates induced a dose-dependent cytotoxicity from either side of the monolayer. Since the first significant, cytotoxic effect could be observed after basolateral treatment of the monolayers with a concentration of both conjugates as low as 500 μM (unpublished results), this concentration was chosen throughout the experiments. When indicated, monolayers were pre-incubated with either 500 μM acivicin, amino-oxyacetic acid (AOAA) or probenecid (Mertens *et al.*, 1988) or 1 mM paraoxon (Neis *et al.*, 1985) for half an hour. Following exposure, cells were left in WE-medium supplemented with gentamycin. After an overnight incubation, the apical and basolateral media

were combined and cytotoxicity was determined by measuring LDH-leakage (Mertens *et al.*, 1988).

Statistics

Data are presented as the means (± SEM) of at least three experiments in triplicate. For statistical evaluation the Student's *t*-test, one tailed, was applied. Significancy is indicated by asterisks. One symbol indicates $P \leq 0.05$; two symbols $P \leq 0.02$; three and four symbols $P \leq 0.01$ and $P \leq 0.001$, respectively.

RESULTS

Confluency of monolayers

After four days of culture, approximately 90% of the monolayers were confluent. Maximal confluency could only be kept for approximately one day. Thereafter, monolayers became more and more leaky and cell viability gradually declined. Under the culture conditions used, confluent RPT cell monolayers displayed a net TEER of about 400 Ohm/cm^2. Therefore, experiments were always performed with four-day-old cultures, displaying a TEER of minimally 400 Ohm/cm^2.

Cytotoxicity of MGNQ and M(NAC)NQ

A marginal but significant cytotoxic effect could be observed only after basolateral exposure of RPT cell monolayers to MGNQ (Fig. 1A). Whereas the basolaterally induced LDH-leakage was 24.8 ± 4.0%, the apically-induced LDH-leakage was at the control level (16.3 ± 1.5%).

To find out whether γGT activity plays a role in the detoxification of MGNQ after apical challenge with MGNQ, we pre-incubated monolayers with acivicin, an inhibitor of γGT (Fig. 2A). Indeed, inhibition of γGT activity resulted in the appearance of a toxic effect after apical exposure of RPT cells to MGNQ. MGNQ-induced LDH-leakage from RPT cell monolayers pre-incubated with acivicin was almost two-fold higher as compared to control cells or cells treated with MGNQ without acivicin.

In contrast to MGNQ, M(NAC)NQ was found to be toxic both after apical and basolateral challenge of the monolayer. Furthermore, after basolateral exposure LDH-leakage was twofold higher than after apical exposure (35.8 ± 3.6% and 17.5 ± 0.9%, respectively) (Fig. 1B).

To establish the role of the basolaterally located organic anion transport system in the toxicity of MGNQ and M(NAC)NQ, we pre-incubated RPT cells with probenecid. Simultaneous basolateral exposure of these cells to MGNQ or M(NAC)NQ and probenecid, both resulted in a

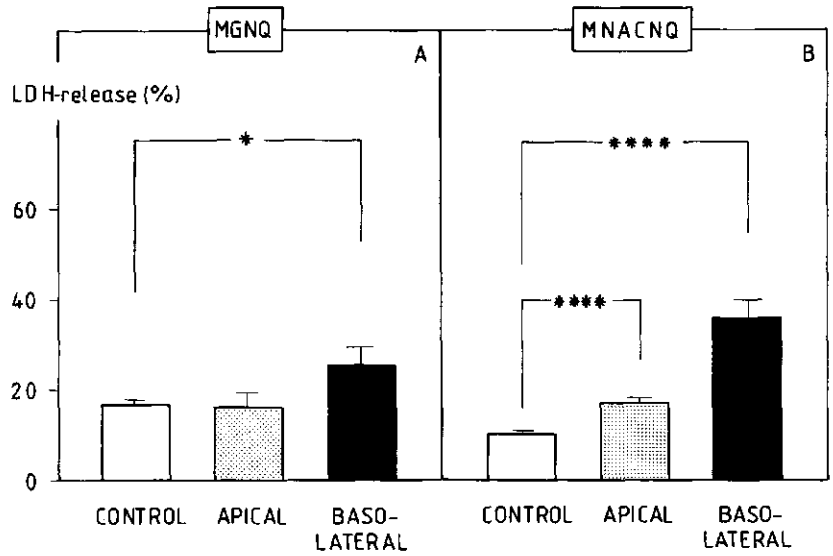

Figure 1. Effect of apical and basolateral exposure of RPT cell monolayers to MGNQ (A) and M(NAC)NQ (B). Confluent RPT cell monolayers were exposed apically or basolaterally to 500 μM MGNQ or M(NAC)NQ in EBSS for 4h. After this period, the medium was changed and monolayers were kept in WE-medium. Toxicity was measured by LDH-leakage after an overnight incubation. Each value represents the mean (± SEM) of three experiments in triplicate.

potentiation of the toxic effect to a comparable value (Fig. 2B and 3A, respectively). Cellular leakage of LDH after MGNQ challenge only was 34.1 ± 4.3%. However, the MGNQ-induced LDH-leakage from probenecid pre-treated monolayers was 55.0 ± 3.2%. Similarly, LDH-leakage from M(NAC)NQ-treated tissue was lower (41.7 ± 4.6%) than the M(NAC)NQ-induced LDH-leakage from the probenecid pre-treated tissue (60.6 ± 5.1%).

As mercapturic acids taken up basolaterally may be transformed into toxic thiols, we studied the contribution of this pathway to the observed toxicity. Firstly, inhibition of β-lyase activity by pre-incubating RPT cell monolayers with AOAA did not alter M(NAC)NQ-induced toxicity after basolateral exposure (Fig. 3B). While the M(NAC)NQ-induced LDH-leakage from AOAA pre-incubated tissue was 48.1 ± 7.0%, LDH-leakage after M(NAC)NQ exposure alone was of the same order of magnitude (43.0 ± 7.7%). Secondly, we inhibited the *N*-deacetylation activity of the cell with a non-toxic concentration of paraoxon. Whereas both the control-treated and the paraoxon-treated monolayers displayed an LDH-leakage of about 11%, LDH-leakage after

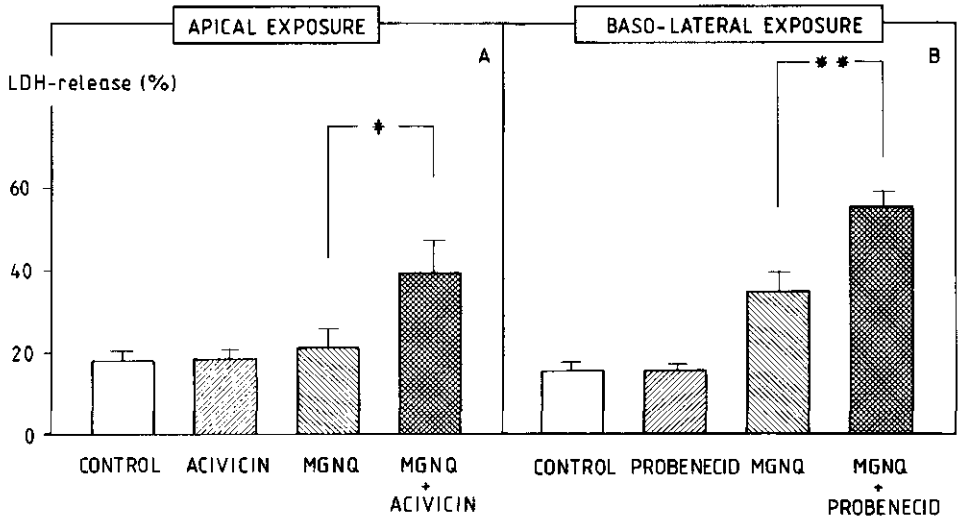

Figure 2. Effect of acivicin and probenecid on MGNQ-induced cytotoxicity. Confluent monolayers were apically pre-treated with (A) 500 μM acivicin or basolaterally with (B) 500 μM probenecid in EBSS for 30 min. After this period, monolayers were treated apically with 500 μM MGNQ or basolaterally with 500 μM MGNQ in the presence of probenecid in EBSS for 4h. Thereafter, medium was changed and monolayers were kept in WE-medium. After an overnight incubation, LDH-leakage was measured. Each value represents the mean (± SEM) of three experiments in triplicate.

M(NAC)NQ exposure from paraoxon pre-treated cells was significantly higher (51.9 ± 5.6%) than from M(NAC)NQ-treated cells (31.7 ± 3.2%) (Fig. 3C).

DISCUSSION

This *in vitro* study is the first to describe proximal tubular toxicity by quinone-thioethers after incubation with these compounds on either side of a RPT cell monolayer.

MGNQ was found to be toxic after basolateral challenge, but not after apical challenge. In a suspension of renal epithelial cells Brown *et al.* (1991) observed no cellular toxicity of MGNQ. Our results and those of Brown *et al.* (1991) are in agreement with observations by Lau *et al.* (1990), who did not find histological alterations in the kidney after *in vivo* exposure of rats to MGNQ. It has been established that GSH conjugates require enzymatic processing by γGT in

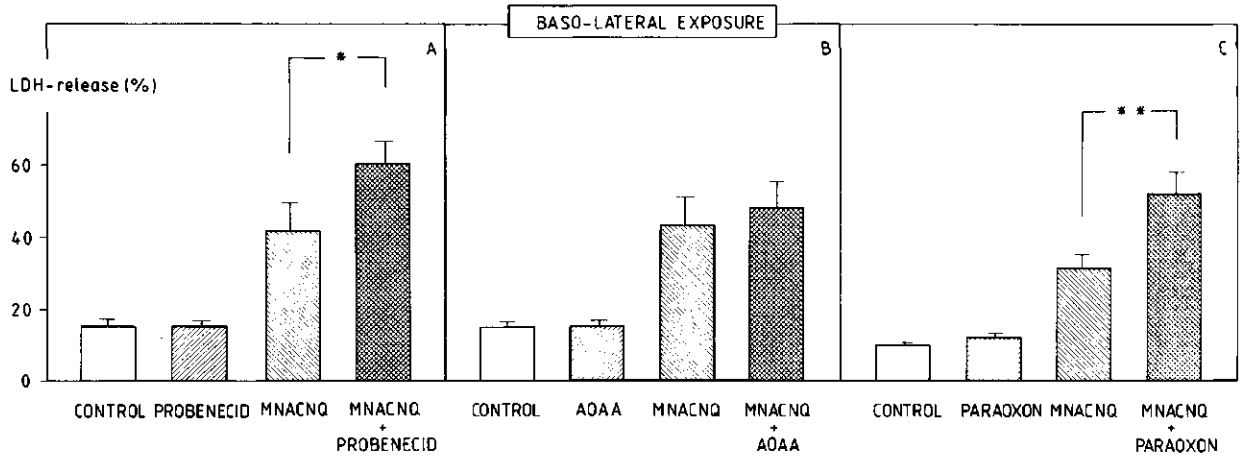

Figure 3. *Modulation of M(NAC)NQ-induced cytotoxicity after basolateral exposure. Confluent monolayers were basolaterally pre-treated for 30 min with (A) 500 μM probenecid, or apically and basolaterally with (B) 500 μM amino-oxyacetic acid (AOAA) or (C) 1 mM paraoxon in EBSS. Medium in the basolateral chamber was changed and cells were basolaterally exposed to 500 μM M(NAC)NQ in the presence or absence of probenecid (A), AOAA (B) or paraoxon (C) in EBSS for 4h. After an overnight incubation in WE-medium, LDH-leakage was measured. Each value represents the mean (± SEM) of four experiments in triplicate.*

order to facilitate uptake into the proximal tubular cells (Curthoys and Hughey, 1979). Recently, Monks *et al.* (1990a) provided evidence that the γGT-catalyzed processing of GSH conjugates initiates a novel detoxification reaction. This pathway involves the intramolecular cyclization of either the cysteinylglycine and/or the cysteine conjugate to give a 1,4-benzothiazine derivative lacking the reactive quinone function. As a consequence of this, these compounds appear to be non-toxic *in vivo* as well (Monks *et al.*, 1990b; Mertens *et al.*, 1991). However, it remains to be established firmly that 1,4-benzothiazines are actually formed in kidney tissue *in vivo*. The fact that urine samples of rats exposed to GSH conjugates of chlorohydroquinones displayed a black color is suggestive for the formation of these products *in vivo* (Mertens *et al.* (1991).

In support of this interpretation we showed that pre-treatment of RPT cells with acivicin, an inhibitor of several glutamine-utilizing enzymes (Weber, 1983) including γGT (Reed *et al.*, 1980), resulted in the appearance of a toxic effect of MGNQ. Since γGT-mediated cleavage of the GSH portion of quinones is a prerequisite for cellular uptake of the conjugate, the observed toxicity of MGNQ after γGT inhibition seems to be of extracellular origin. The fact that MGNQ induced a marginal but significant cytotoxicity after basolateral challenge is in line with the fact that basolaterally there is much less γGT activity than apically (Spater *et al.*, 1982). The lack of detoxification by γGT presumably accounts for the observed toxicity. The acivicin-induced stimulation of quinol-GSH toxicity has also been observed in *in vivo* studies when 2,5-dichloro-1,4-benzoquinone and 2,5,6-trichloro-1,4-benzoquinone were given to rats in the presence of ascorbic acid (Monks *et al.*, 1990a; Mertens *et al.*, 1991).

In contrast to MGNQ, apical treatment of RPT cells monolayers with M(NAC)NQ induced a significant toxic effect. In addition, the basolaterally induced cytotoxicity by M(NAC)NQ was even two-fold higher. In our proximal tubular cells the organic anion transporter is present (Lash and Anders, 1989; Bruggeman *et al.*, 1989). Therefore, one might conclude that the difference in toxicity after basolateral and apical exposure is associated with basolateral uptake of M(NAC)NQ. On the other hand, simultaneous basolateral exposure of RPT cells to MGNQ or M(NAC)NQ and probenecid, an inhibitor of the organic anion transport system (Lash and Jones, 1985), resulted in a potentiation of the toxic effects. These results suggest an extracellular component in the toxicity of these conjugates. This conclusion, however, is in contrast with results of Redegeld *et al.* (1991), who observed a significant decline in cytotoxicity by MGNQ after pre-incubation of isolated, perfused kidneys with acivicin and probenecid. The difference in *in vitro* models used to study MGNQ-induced nephrotoxicity may account for this apparent discrepancy in effects.

The higher level of toxicity after basolateral challenge of RPT cells with M(NAC)NQ as compared with apical challenge (Fig. 1B) may be the consequence of apically located targets for M(NAC)NQ-induced toxicity being less critical for cell viability than those situated basolaterally. Alternatively, the apical membrane might contain unknown detoxification mechanisms with a

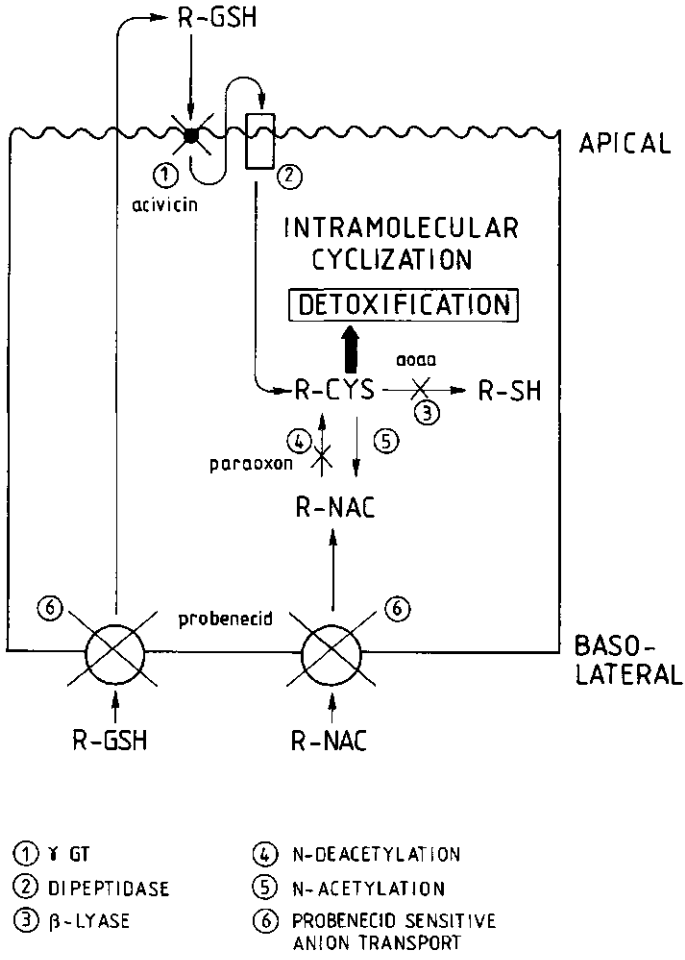

Figure 4. *Postulated reaction mechanism resulting in detoxification of R-GSH and R-NAC conjugates in RPT cells. R-GSH can enter the RPT cell from the apical side via a γGT and dipeptidase-mediated metabolism. Cellular uptake of R-GSH and R-NAC from the basolateral side is presumed to take place for most part via a probenecid-sensitive anion transport system. Uptake of R-GSH or R-NAC results in the formation of a cysteine conjugate that loses its redox cycling capacity, presumably via intramolecular cyclization. Blocking cellular entry of the conjugates from either apical (acivicin) or basolateral (probenecid) side, results in a high extracellular concentration of conjugates. As these conjugates possess redox cycling capacity (Brown et al., 1991), it is possible that they induce lipid peroxidation of cell membranes. In addition, both conjugates may interact with thiol groups of extracellularly located functional proteins which play a critical role in cell function.*

higher detoxifying capacity than the basolaterally located detoxifying mechanisms. Thirdly, cellular toxicity of basolaterally applied MGNQ and M(NAC)NQ (Figs. 1B, 2B and 3) may in part be the result of cellular uptake by organic anion carriers and subsequent (intracellular) redox cycling without sufficient cyclization. This seems plausible, since cells cultured at a high artificial oxygen pressure may contain higher oxygen levels than under more physiological conditions.

N-acetylcysteine conjugates once taken up, are deacetylated intracellularly to generate the cysteine conjugate (Suzuki and Tateishi, 1981; Commandeur and Vermeulen, 1990). Since intramolecular cyclization of quinol-linked cysteine-*S*-yl conjugates appear to constitute a detoxification reaction, it seems very likely that M(NAC)NQ after deacetylation to M(CYS)NQ will also be detoxified. Indeed, inhibition of the deacetylation reaction with paraoxon prevented detoxification of M(NAC)NQ and potentiated its effect.

In line with other studies on quinones (Monks *et al.*, 1988; Lau *et al.*, 1988), inhibition of β-lyase did not attenuate toxicity, suggesting that, in contrast to haloalkenes, toxic thiols do not play a role in nephrotoxicity of quinol-thioethers.

A schematic summary of the putative reaction mechanism resulting in detoxification of M(NAC)NQ and MGNQ ending with cyclization, is shown in Fig. 4. The observation that intramolecular cyclization occurs not only with cysteine *S*-conjugates of benzo- and hydroquinones but also with naphthoquinones like 1,4-naphthoquinone (Kuhn and Hammer, 1951), supports this concept.

In conclusion, our results suggest that cellular uptake of conjugates either via the apical or basolateral membrane is associated with detoxification. Blocking entry of MGNQ or M(NAC)NQ into RPT cells results in an increased cytotoxicity. Whether presumed extracellular events, like oxidation of thiol groups of membrane proteins or lipid peroxidation of cell membranes, account for the observed increase in cytotoxicity, requires further investigation.

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CHAPTER 3

Cytotoxicity of 2-*tert*-butyl hydroquinone glutathione conjugates after apical and basolateral exposure of rat renal proximal tubular cell monolayers

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SUMMARY

Confluent monolayers of renal proximal tubular (RPT) cells cultured on porous supports were used to investigate the cytotoxicity induced by glutathione conjugates of 2-*tert*-butyl-(1,4)-hydroquinone (SG-TBHQ) after apical and basolateral exposure. As judged from lactate dehydrogenase (LDH) -leakage, cytotoxicity was observed after basolateral exposure of monolayers to 250 and 500 μM of 2-*tert*-butyl-5-(glutathion-*S*-yl)hydroquinone (5SG-TBHQ). In these experiments, LDH-leakage was 22.3 $\pm$ 1.9% and 32.2 $\pm$ 1.9%, respectively. Basolateral exposure of monolayers to 250 and 500 μM 6SG-TBHQ resulted in LDH-leakage of 22.2 $\pm$ 2.5% and 30.0 $\pm$ 2.7%, respectively. The double conjugate, 2-*tert*-butyl-3,6-(diglutathione-*S*-yl)hydroquinone (3,6SG-TBHQ), was not toxic and LDH-leakage was about control level (15.0%). Basolaterally located, probenecid-sensitive organic anion transporters did not seem to play a role in the cytotoxic effect. However, when RPT cell monolayers were cultured in 24-well tissue culture plates, apical challenge with 250 μM of 5SG-TBHQ induced a cytotoxic effect. In these experiments, LDH-leakage was 33.5 $\pm$ 0.6%. With these cells, inhibition of apical γglutamyltranspeptidase (γGT) -activity by acivicin, which was not toxic by itself, decreased 5SG-TBHQ induced LDH-leakage to 19.3 $\pm$ 1.2%, whereas 250 μM 6SG-TBHQ induced LDH-leakage was increased to 55.3 $\pm$ 1.0%. Co-incubation of RPT cells with SG-TBHQs in the presence of 1.5 mM ascorbic acid (AA) pointed to a pro-oxidant rather than an antioxidant effect of AA. Superoxide dismutase (SOD) and catalase (CAT) completely abolished SG-TBHQ induced cytotoxicity. Since cultured RPT cells lack *N*-acetylation of cysteine conjugates (Haenen *et al.*, 1995), the *N*-deacety,ation/*N*-acetylation ratio cannot play a vital role in renal toxicity of quinone-thioethers in this *in vitro* system. It seems therefore that the cytotoxicity observed is mainly the result of extracellular redox cycling of SG-TBHQs. The lack of toxicity of 6SG-TBHQ after apical exposure could be due to detoxification by γGT-mediated cysteinylglycine- or

cysteine-conjugate formation followed by cyclization, as shown for other related quinone glutathione conjugates. The relative importance of the observed effects for the *in vivo* situation is discussed.

INTRODUCTION

2-*tert*-Butyl-4-hydroxyanisole (BHA) is a phenolic food antioxidant that is used to prevent oxidative deterioration of fats, oils and other ingredients. Although BHA is not carcinogenic in humans, carcinogenic and toxic effects of BHA were observed in experimental animals. In the forestomach of rodents, BHA induces papillomas and carcinomas, preceded by an increase in cellular proliferation (for review, see Verhagen *et al.*, 1991). In rodents, the BHA-induced cell proliferation-enhancing effects were also found in other tissues of epithelial origin, including renal epithelia (Amo *et al.*, 1990; Lau *et al.*, 1994).

Although the toxic and carcinogenic effects of BHA are well described, little is known about the mechanism by which this antioxidant causes toxicity and carcinogenicity. It is generally accepted that the ultimate reactive forms of carcinogens are electrophilic species. Since BHA is not electrophilic in nature, it has to be metabolised to an electrophilic compound. From *in vitro* (Rahimthula, 1983; Cummings *et al.*, 1985) and *in vivo* studies (Astill *et al.*, 1962; El-Rashidy and Niazi, 1983) it was concluded that the main metabolic transformation of BHA comprises *O*-demethylation yielding 2-*tert*-butyl-(1,4)-hydroquinone (TBHQ). By oxidation of the latter compound, 2-*tert*-butyl-(1,4)-quinone (TBQ) is formed. TBQ is a strong electrophile and, as a result of redox cycling, TBQ and TBHQ can generate reactive oxygen species via semiquinone radicals leading to an oxidative stress (Kahl *et al.*, 1989; Van Ommen *et al.*, 1992). In this context, TBHQ and TBQ are far more potent in stimulating superoxide production in microsomes than BHA itself (Kahl *et al.*, 1989).

Owing to their electrophilic nature, quinones can conjugate with sulfhydryl compounds like glutathione (GSH), which is the most abundant cellular non-protein sulfhydryl (Reed and Meredith, 1985). GSH appears to activate BHA since co-administration of BHA and the GSH-depleting agent diethylmaleate completely inhibited BHA-induced forestomach hyperplasia in rat (Hirose *et al.*, 1987). In addition, in both *in vivo* and *in vitro* experiments, two GSH conjugates of TBHQ (5GS-TBHQ and 6GS-TBHQ) were identified (Tajima *et al.*, 1991). A third, diGS-TBHQ conjugate (3,6SG-TBHQ), was identified by Peters *et al.* (1996).

It is generally accepted that GSH-conjugation of (hydro)quinones often results in the formation of potent and selective (nephro)toxicants (Monks and Lau, 1992). In line with this,

GSH conjugates of TBHQ might be the reactive intermediates of BHA-induced (nephro)carcinogenicity, since these conjugates were far more potent in generating reactive oxygen species than TBHQ or BHA (Van Ommen *et al.*, 1992; Schilderman *et al.*, 1993). Evidence for a key role of GSH-conjugated TBHQ in the promotion of nephrocarcinogenicity by BHA was provided by Lau and co-workers (1994). In addition, GSH conjugates of TBHQ were found to induce selective proximal tubular damage (Lau *et al.*, 1994).

Therefore, the present study was undertaken to investigate proximal tubular toxicity of 5, 6 and 3,6SG-TBHQ in more detail. *In vivo*, RPT cells can be exposed apically to glomerulary-filtrated and basolaterally to non-filtrated conjugates. Using confluent monolayers of rat RPT cells cultured on porous supports of tissue culture inserts, we determined the relative contribution to proximal tubular damage of apically versus basolaterally induced cytotoxicity by these conjugates.

MATERIALS AND METHODS

Chemicals

2-*tert*-Butyl-(1,4)-hydroquinone was obtained from Aldrich (Steinheim, Germany). GSH, acivicin, probenecid, superoxide dismutase and catalase (from bovine liver) were from Sigma Chemical Co. (St Louis, MO, USA). Ascorbic acid (AA) was bought from Merck (Darmstadt, Germany). Collagenase (from *Clostridium histolyticum)* was purchased from Boehringer (Mannheim, Germany). Nycodenz (iohexol) was obtained from Nycomed AS (Oslo, Norway). All other reagents were of the highest grade commercially available.

Syntheses of GSH conjugates

2-*tert*-Butyl-5-(glutathion-*S*-yl)hydroquinone (5SG-TBHQ), 2-*tert*-butyl-6-(glutathion-*S*-yl)hydroquinone (6GS-TBHQ) and 2-*tert*-butyl-3,6-(diglutathion-*S*-yl)hydroquinone (3,6SG-TBHQ) were synthesized as described by Van Ommen *et al.* (1992). In brief, TBQ was obtained by oxidation of TBHQ using potassium bromate in 1 N sulfuric acid. To a solution of 137 mg TBQ in methanol an aqueous solution of 256 mg GSH was added. This reaction mixture was stirred for 22h under N_2. After reduction of all constituents in the reaction mixture with AA, the conjugates formed were purified by preparative high performance liquid chromatography (HPLC). For purification, a Zorbax 22x250 mm RP18 column was eluted with 55% water containing 0.5% formic acid and 45% methanol at a flow rate of 5 ml/min. Diode array detection (Pharmacia Rapid Spectra 2140) provided information on retention times and spectra of the

various reaction products. Structural characterization of the products was performed by Fast Atom Bombardment Mass Spectrometry, positive ion detection (Finigan-Matt HSQ-30, BEQQ geometry) and proton NMR using a Varian Unity-400 spectrometer.

Cell isolation and culture

For isolation of RPT cells, female Wistar rats (Harlan CPB, Zeist, The Netherlands) weighing 180-230 g were used. RPT cells were isolated according to a method by Boogaard *et al.* (1989). RPT cells were seeded on collagen-coated tissue culture inserts (Costar, Badhoevedorp, The Netherlands). Cells were cultured in Williams' E Medium (WE) (Flow, Irvine, Scotland) supplemented with 10% foetal calf serum (Gibco, Glasgow, Scotland) and gentamycin (50 μg/ml) (Gibco, Paisley, Scotland) at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity assay

After 4 days of culture more than 90% of the RPT cell monolayers were confluent. For experiments, only monolayers displaying a net transepithelial electrical resistance (TEER) of more than 400 ohm/cm^2 were used (Haenen *et al.*, 1994). Monolayers were washed with Earls' Balanced Salt Solution (EBSS, pH 7.4) supplemented with 10 mM $NaHCO_3$ and 20 mM HEPES. RPT cell monolayers were apically or basolaterally challenged with SG-TBHQ for 4h in EBSS. When indicated (see figure legends) this time period was preceded by a pre-incubation period of 30 min. Following exposure, RPT cell monolayers were left overnight in WE medium supplemented with gentamycin. After this period, apical and basolateral media were combined and cytotoxicity was assessed by measuring lactate dehydrogenase (LDH)-leakage (Mertens *et al.*, 1988).

Statistics

Data are presented as the means ($\pm$ SEM) of at least three experiments in triplicate. Statistical comparisons were made between groups using the Student's *t*-test (one tailed). Significance is indicated by asterisks. One symbol indicates $P \leq 0.05$; two symbols $P \leq 0.02$; three and four symbols $P \leq 0.01$ and $P \leq 0.001$, respectively.

RESULTS

Apical exposure of RPT cell monolayers cultured on porous supports to 125-500 μM 5, 6, and 3,6SG-TBHQ conjugate for 4h did not result in a cytotoxic effect (Fig. 1). All concentrations of

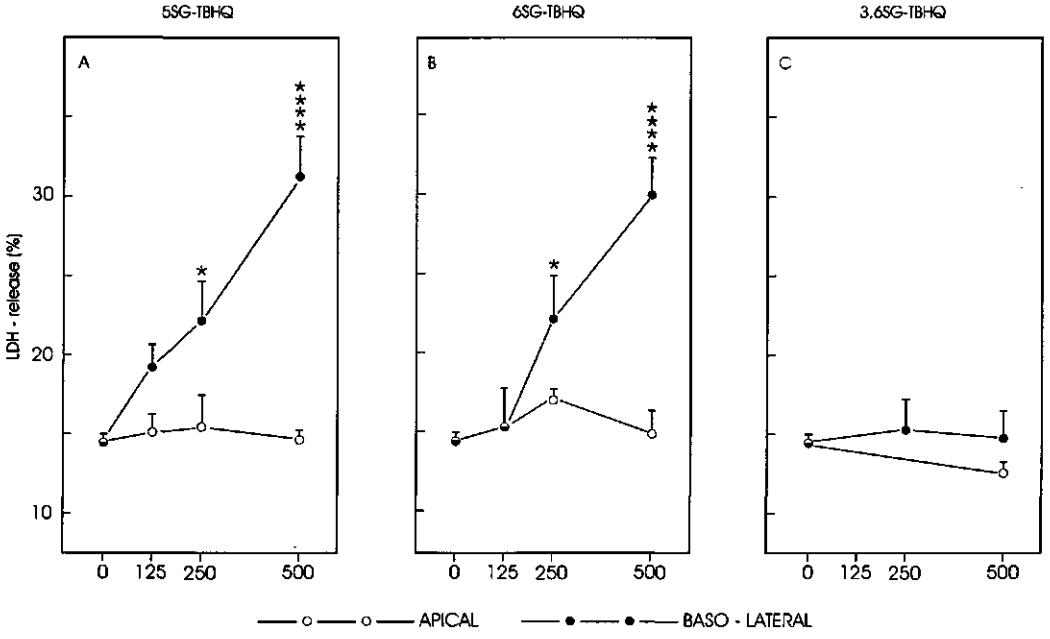

Figure 1. Apical and basolateral toxicity of 5SG-TBHQ (A), 6SG-TBHQ (B) and 3,6SG-TBHQ (C) conjugates. Confluent monolayers of RPT cells grown on porous supports of tissue culture inserts were exposed apically or basolaterally to 125, 250 and 500 μM of conjugate in EBSS for 4h. After this period, the medium was changed and monolayers were kept overnight in WE medium. Toxicity was measured by means of LDH-leakage. Data are the mean (± SEM) of at least four experiments.

conjugate applied induced LDH-leakage of about control level (15.3 ± 0.8%).

However, after basolateral exposure of monolayers to 250 μM 5SG-TBHQ (A) and 6SG-TBHQ (B), a significant increase in LDH-leakage to 22.3 ± 1.9% and 22.2 ± 2.5%, respectively, was observed. After basolateral challenge of monolayers to 500 μM of the mono-substituted conjugates, cellular toxicity was increased to 32.2 ± 1.9% and 30.0 ± 2.7% for 5SG-TBHQ and 6SG-TBHQ, respectively. No basolaterally induced cytotoxicity was observed after exposure of RPT cells to 3,6SG-TBHQ (C).

To find out whether γGT plays a role in the detoxification of apically applied SG-TBHQ, we pre-incubated RPT cell monolayers cultured in 24-well tissue culture plates with acivicin, an inhibitor of γGT (Fig. 2). Acivicin was not cytotoxic and the observed LDH-leakage was only 12.6 ± 0.9%. In these experiments, 5SG-TBHQ induced a dose-dependent cytotoxicity (results

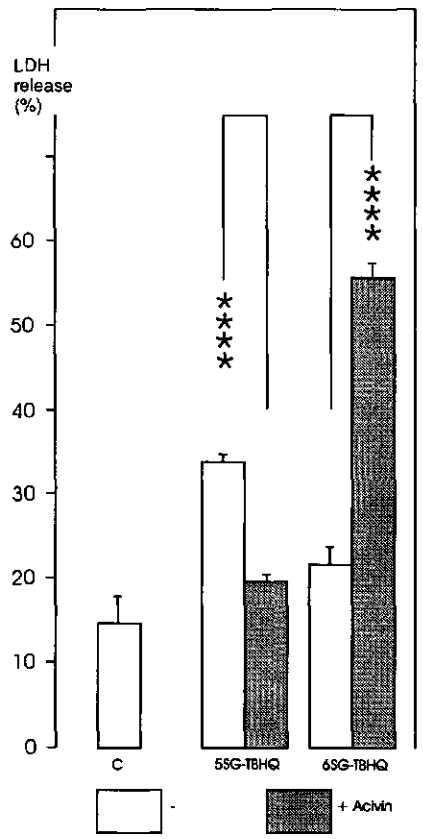

Figure 2. Effect of acivicin pre-treatment on apical exposure of RPT cells to 5SG-TBHQ and 6SG-TBHQ. RPT cells were seeded in 24-well tissue culture plates at a density of 1.10^5 cells/cm^2. After three days of culture, monolayers were formed and experiments were started. RPT cell monolayers were pretreated with 500 μM acivicin for 30 min. After this period, monolayers were treated with 250 μM 5SG-TBHQ or 6SG-TBHQ in EBSS for 4h. Thereafter, the medium was changed and monolayers were kept in WE medium. After an overnight incubation, LDH-leakage was measured. Each value represents the mean (± SEM) of four experiments. The 5SG-TBHQ induced LDH-leakage is significantly different from control LDH-leakage ($p \leq 0.001$). Acivicin induced an LDH-leakage of 12.6 ± 0.9%.

not shown). At 250 μM, 5SG-TBHQ induced an LDH-leakage of 33.5 ± 0.6% which was significantly different from control level (14.5 ± 3.0%). Similarly, 250 μM 6SG-TBHQ induced an LDH-leakage of 21.7 ± 1.9%. Surprisingly, acivicin pre-treatment significantly reduced the LDH-leakage by 5SG-TBHQ (19.3 ± 1.2%), whereas a significant increase in cytotoxicity was observed after incubation of these cells with 6SG-TBHQ (55.3 ± 1.0%). No such effect of acivicin could be observed with RPT cells cultured on porous supports.

The role of basolaterally located organic anion transporters in the basolaterally induced cytotoxicity of the SG-TBHQ conjugates was established by pre-incubating RPT cell monolayers cultured on porous supports with probenecid (Fig. 3). Simultaneous basolateral exposure of these monolayers with probenecid and 500 μM of SG-TBHQ conjugate did not have an effect on conjugate-induced basolateral cytotoxicity.

To investigate the effect of SG-TBHQ conjugates upon RPT cell monolayers without the

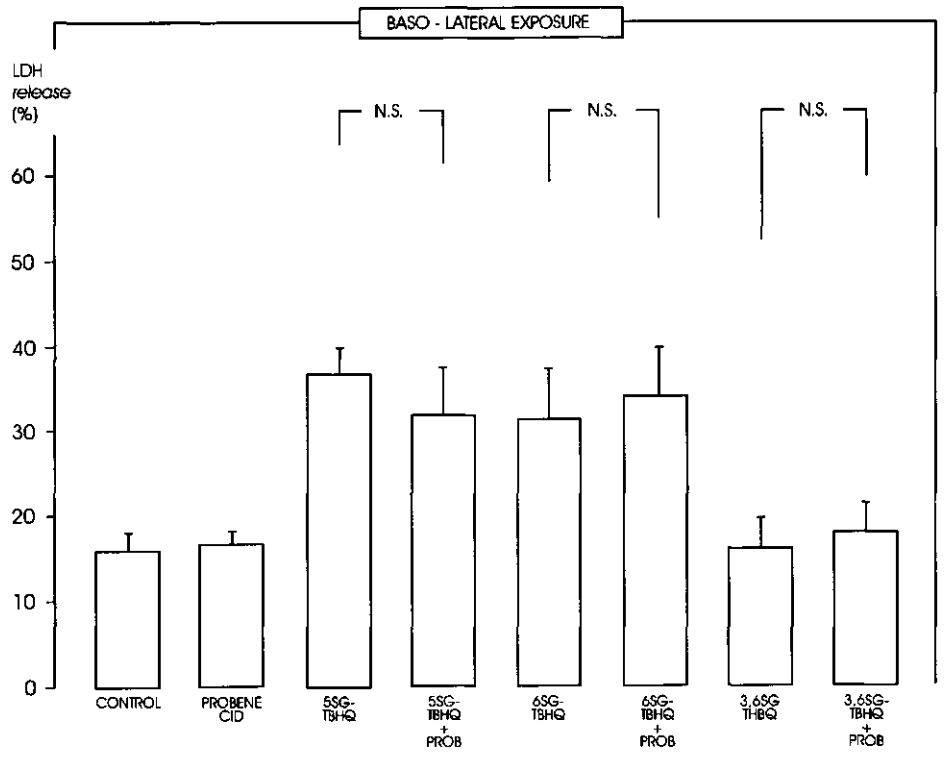

Figure 3. *Effect of probenecid on the basolaterally-induced cytotoxicity by 5SG-TBHQ, 6SG-TBHQ and 3,6SG-TBHQ. Monolayers were basolaterally pre-incubated with 500 μM probenecid in EBSS for 30 min. After this period, monolayers were basolaterally exposed to 500 μM of SG-TBHQ conjugate or to conjugate in the presence of probenecid in EBSS for 4h. Following this, medium was changed and monolayers were kept in WE medium. After an overnight incubation, LDH-leakage was measured. Bars represent mean (± SEM) of four experiments.*

interference of concomitant (extracellular) redox-cycling, we administered the hydroquinone conjugate with a threefold molar excess of AA (Mertens *et al.*, 1991). As compared to control-treated cells, pre-incubation of monolayers cultured on porous supports with 1.5 mM AA induced a significant increase in LDH-leakage after apical challenge of this tissue with 500 μM 5, 6 and 3,6SG-TBHQ in the presence of AA to approximately 25% (Fig. 4). Apically applied SG-TBHQ conjugates and AA alone induced LDH-leakage of about control level (13.0 ± 0.5%). Basolateral co-incubation of AA-pretreated monolayers with SG-TBHQ conjugates and AA also

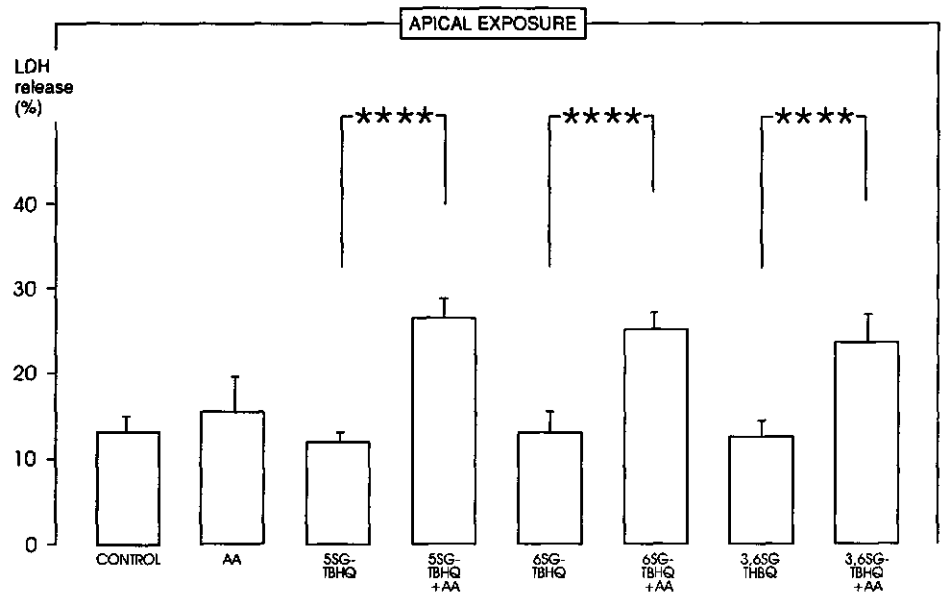

Figure 4. *Effect of AA on the apical challenge of RPT cell monolayers to 5SG-TBHQ, 6SG-TBHQ and 3,6SG-TBHQ. Monolayers were apically pre-treated with 1.5 mM ascorbic acid (AA) in EBSS for 30 min. After this period, monolayers were apically challenged with 500 μM of SG-TBHQ conjugate or with SG-TBHQ conjugate in the presence of AA in EBSS for 4h. Afterwards, medium was changed and monolayers were kept in WE medium. After an overnight incubation, LDH-leakage was measured. Bars represent the mean (± SEM) of at least four experiments.*

resulted in a significant increase in cellular toxicity as compared to control-treated tissue (Fig. 5). In the presence of AA, the 5SG-TBHQ and 6SG-TBHQ induced LDH-leakage was of the same magnitude (58.2 ± 2.6% and 55.2 ± 2.9%, respectively), whereas the 3,6 SG-TBHQ-induced LDH-leakage was 44.4 ± 2.8%.

To find out whether extracellular redox cycling was responsible for the observed proximal tubular toxicity by the SG-TBHQ conjugates and AA, RPT cell monolayers were challenged with 5SG-TBHQ and AA, or with 5SG-TBHQ and AA in the presence of superoxide dismutase (SOD: 20 μg/ml) and catalase (CAT: 3 U/ml) (Fig. 6). Apical challenge of monolayers with 750 μM of conjugate in the presence of 1.5 mM AA induced an LDH-leakage of 73.9 ± 3.6%. Basolateral exposure of RPT cell monolayers to 125 μM conjugate in the presence of AA induced an LDH-leakage of 55.9 ± 7.1%. After apical or basolateral challenge of these monolayers to 5SG-TBHQ

and AA in the presence of SOD and catalase no cytotoxicity was observed.

DISCUSSION

In this *in vitro* study, cytotoxicity could only be monitored after basolateral challenge of RPTC monolayers on porous supports with both mono-substituted SG-TBHQ conjugates. When cultured on solid supports of 24-well plastic tissue culture plates a toxic effect of 5SG-TBHQ could only be seen on the apical side. These results are consistent with an earlier study in which thioethers of menadione were also more toxic to RPT cell monolayers after basolateral challenge than after apical challenge (Haenen *et al.*, 1994). An explanation for this difference in cytotoxicity could be that basolaterally located targets are more critical for cell viability than those on the apical membrane. Probenecid sensitive organic anion transporters do not seem to play a role in basolaterally induced cytotoxicity by 5- and 6SG-TBHQ conjugates, since simultaneous basolateral challenge of RPT cell monolayers to probenecid and either 5SG-TBHQ or 6SG-TBHQ did not affect the observed cytotoxicity by the conjugate alone. However, these transport systems did seem to play a role in basolateral uptake of thioethers of menadione (Haenen *et al.*, 1994).

We observed that mono-substituted GSH conjugates of TBHQ are toxic in this *in vitro* system whereas di-substituted GSH conjugates are not. This result is in agreement with the higher redox cycling activity of both 5SG-TBHQ and 6SG-TBHQ compared to 3,6SG-TBHQ by Van Ommen *et al.* (1992). Since cultured RPT cells lack the capacity to *N*-acetylate cysteine conjugates (Haenen *et al.*, 1995), the *N*-deacetylation/*N*-acetylation ratio cannot play a vital role in renal toxicity of quinone-thioethers in this *in vitro* system like it seems to play *in vivo* (Monks *et al.*, 1994). In addition, compared to the *in vivo* situation, cell culture is carried out at an artificially high oxygen pressure (20%). Under these conditions, extensive redox cycling of (hydro)quinones is to be expected. As a result, the magnitude of redox cycling of (hydro)quinones *in vitro*, and thus the extent of quinone-thioether toxicity, may not reflect redox cycling of (hydro)quinones *in vivo*.

What role does γGT play in this context? Evidence is accumulating that γGT on the one hand initiates detoxification of GSH conjugated (hydro)quinones by formation of the cysteinylglycine conjugate followed by cyclization of the conjugate, thus removing the reactive quinone function. On the other hand γGT gives rise to conjugates that can undergo redox cycling more easily (reviewed in Monks and Lau, 1992). However, γGT activity does not seem to play a critical role in quinone-GSH induced renal toxicity *in vivo* (Hill *et al.*, 1991; Monks *et al.*, 1994). In our *in vitro* system, 3,6SG-TBHQ did not induce cytotoxicity. Probably, this conjugate

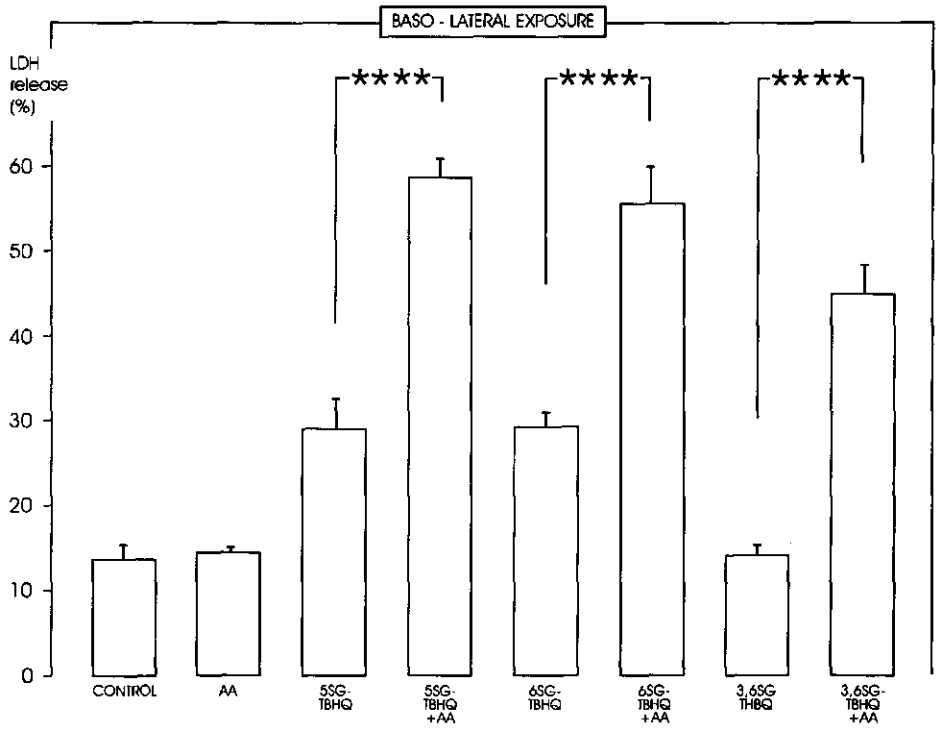

Figure 5. Effect of AA on the basolateral challenge of renal proximal tubular cell monolayers to 5SG-TBHQ, 6SG-TBHQ and 3,6SG-TBHQ. Monolayers were basolaterally pretreated with 1.5 mM AA in EBSS for 30 min. After this pre-incubation period, monolayers were basolaterally challenged with 500 μM of SG-TBHQ conjugate or with SG-TBHQ conjugate in the presence of AA in EBSS for 4h. After this period, EBSS medium was changed and replaced by WE medium. After an overnight incubation, LDH-leakage was measured. Bars represent mean (± SEM) of four experiments.

is a good substrate for γGT leading to complete detoxification. The finding that apical exposure to 5SG-TBHQ was only toxic to confluent RPT cell monolayers cultured in 24-well (plastic) plates and not to confluent monolayers on porous supports suggests that RPT cells cultured on porous supports are less susceptible to this conjugate.

The observation that both 5SG-TBHQ and 6SG-TBHQ are toxic after basolateral challenge of RPT cell monolayers suggests that, at this side of the monolayer, γGT-mediated metabolism of 5- and 6SG-TBHQ is not sufficient for complete detoxification of these conjugates in this *in vitro*

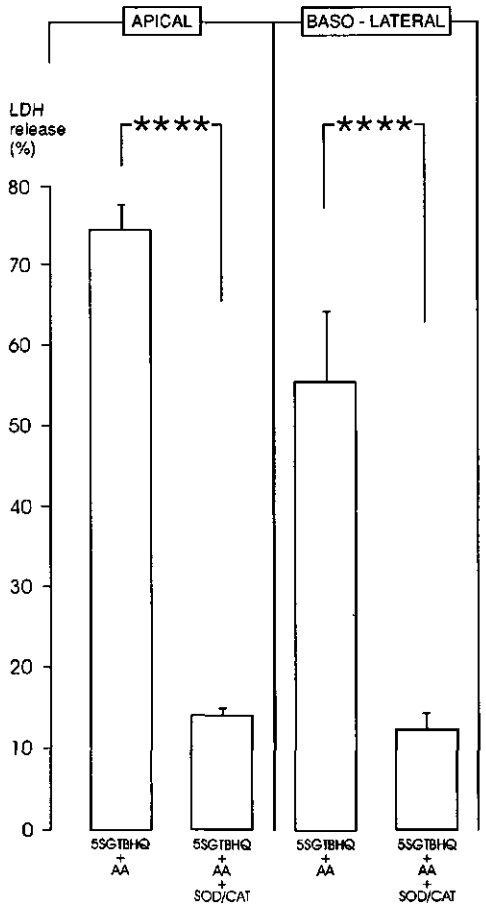

Figure 6. *Effect of SOD and catalase on the apically and basolaterally induced cytotoxicity by a combination of 5SG-TBHQ and AA. Monolayers cultured on porous supports were apically or basolaterally treated with (respectively) 750 or 125 μM 5SG-TBH in the presence of 1.5 mM AA and a combination of SOD (20 μg/ml) and catalase (3 U/ml) for 4h. After this incubation time, monolayers were kept overnight in WE. Cytotoxicity was assessed via LDH-leakage. Bars represent the mean (± SEM) of four experiments. Control LDH leakage was 12.3 ± 0.6%. LDH leakage of AA + SOD/CAT control was 11.0 ± 0.9%*

system. Since AA increased cytotoxicity of basolaterally and apically applied SG-TBHQ conjugates in our study, it is suggested that AA at this concentration acts as a pro-oxidant. It has been reported by Haenen (1989) that at relatively low concentrations AA acts as a pro-oxidant whereas at relatively high concentrations it acts as an antioxidant in an *in vitro* system. It seems therefore that the concentration of AA in the *in vivo* protocol as used by Mertens *et al.* (1991) cannot simply be adopted for *in vitro* cytotoxicity studies in which AA was used to keep hydroquinones in the reduced form. Probably, the concentration of AA used is too low to act as an antioxidant, hence its pro-oxidant activity in this *in vitro* study.

The observed cytotoxicity by SG-TBHQ's and/or AA might be the result of the deleterious effects of reactive oxygen radicals formed during redox cycling of SG-TBHQs. SOD and catalase completely abolished this cytotoxic effect. Because SOD and catalase cannot be transported into

the cell we suggest therefore that the observed cytotoxicity is the result of extracellular redox cycling of SG-TBHQs. However, from experiments in which RPT cells were apically exposed to 5SG-TBHQ and in which acivicin (an inhibitor of γGT activity) decreased toxicity, we conclude that γGT metabolises at least 5SG-TBHQ into a toxic metabolite which acts inside the cell. The relative importance of this phenomenon in renal toxicity *in vivo* remains to be established.

In conclusion, we have shown that *in vitro* renal toxicity of mono- and di-substitu4ed SG-TBHQs differs from *in vivo* cytotoxicity. The exact reason for this discrepancy is unclear. However, differences in metabolic capacity of RPT cells *in vivo* and *in vitro* together with the influence of an artificially high oxygen pressure on redox cycling activity of quinoid compounds makes the usefulness of cultured RPT cells as a tool for investigating *in vivo* toxicity and metabolism of quinoid compounds doubtful.

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CHAPTER 4

Transport and metabolism of glutathione conjugates of menadione and ethacrynic acid in confluent monolayers of rat renal proximal tubular cells

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SUMMARY

Confluent monolayers of primary rat renal proximal tubular (RPT) cells were used to compare transepithelial transport and concomitant metabolism of two different glutathione (GSH) *S*-conjugates. For the GSH-conjugated quinone compound [^{35}S]-GSH conjugated menadione (MGNQ), no specific transepithelial transport was observed. Most likely, [^{35}S]-MGNQ passed the monolayer via paracellular leakage as the result of a reduction in monolayer integrity due to toxicity via extensive redox cycling of the quinone under the culture conditions. RPT cell monolayers metabolise MGNQ into a cysteinylglycine conjugate, which after intramolecular cyclization yields 2*H*-(3-glycinyl)-9-hydroxy-10-methyl-1,4-naphthothiazine. Acivicin, an inhibitor of γglutamyltranspeptidase (γGT), inhibited the formation of this 1,4-naphthothiazine adduct. The second product formed is 1,4-naphthothiazine, formed by loss of glycine via the action of dipeptidases.

Similarly, no basolateral (B) to apical (A) [B⇒A] transport of a GSH-conjugated α,β unsaturated ketone: [^{14}C]-ethacrynic acid (EASG) occurred. However, net transport of [^{14}C]-radioactivity could be observed from A⇒B direction. After 8 hours, 23% of total [^{14}C]-radioactivity was transported from the apical to the basolateral chamber. In both the apical and basolateral chambers free, unconjugated ethacrynic acid (EA) was observed. γGT-mediated metabolism of EASG to the much more unstable cysteinylglycine conjugate leads to relatively large amounts of free EA. Thus, not the GSH conjugate is transported but rather the cysteine adduct and/or free, unconjugated EA. In agreement with this, acivicin reduced A⇒B transport of EASG and inhibited the formation of free EA.

In conclusion, the confluent monolayers of RPT cells do not or no longer possess active basolateral transport systems for GSH conjugates. However, they are still useful for studying biotransformation reactions of thioether conjugates.

INTRODUCTION

The primary organ for clearance of circulating GSH and GSH *S*-conjugates is the kidney (McIntyre and Curthoys, 1980). Although as much as 80% of plasma GSH is cleared by the kidneys (Haberle *et al.*, 1979), only 25% is removed via glomerular filtration, indicating that a significant amount of circulating GSH is cleared via non-filtrating mechanisms involving proximal tubular uptake of GSH across the basolateral membrane (Lash and Jones, 1984). It was concluded by Lash and Jones (1984, 1985) that GSH and GSH *S*-conjugates of trichloroethylene (DCVG: 1,2-dichlorovinylglutathione) are basolaterally taken up via a sodium-coupled and probenecid-inhibitable GSH transporter. GSH *S*-conjugates are excreted in the tubular lumen where they are degraded into the corresponding cysteine *S*-conjugates by γGT and dipeptidases present at the brush border membrane (Inoue and Morino, 1985). Subsequently, uptake of cysteine *S*-conjugates occurs via a sodium-dependent transporter (Schaeffer and Stevens, 1987).

On the other hand, at the basolateral membrane, both sodium-dependent and -independent uptake mechanisms participate in the uptake of cysteine *S*-conjugates (Murer and Gmaj, 1986; Lash and Anders, 1989).

Since γGT and dipeptidase activity were also found in basolateral membranes of proximal tubular cells, basolateral cysteine *S*-transporters do not only transport cysteine *S*-conjugates formed as a result of enterohepatic metabolism of thioethers (Anderson *et al.*, 1980; Spater *et al.*, 1982; Abbott *et al.*, 1984).

Cysteine *S*-conjugates are excreted in the lumen after intracellular *N*-acetylation into their corresponding *N*-acetylcysteine derivatives. Basolateral uptake of *N*-acetylcysteine *S*-conjugates into the renal proximal tubular cells has been shown to take place via a probenecid-inhibitable organic anion system (Zhang and Stevens, 1989).

Most of the information on renal proximal tubular transport of thioether conjugates arises from studies using (halo)alkenes, membrane vesicles, isolated renal tissue and renal cell lines (Lash and Jones, 1984, 1985; Lash and Anders, 1989). In this respect, due to the absence of an organic anion transporter, the usefulness of the $LLCPK_1$ cell line proved to be limited (Mertens *et al.*, 1988; Rabito, 1986). However, for the study of transcellular transport a system is necessary which possesses both apical and basolateral transport systems. In order to find such a system, we studied transport of thioether conjugates using confluent monolayers of rat primary RPT cells cultured on microporous filters. In addition to transport, thioether conjugates are also subjected to γGT and dipeptidase-mediated metabolism at both apical and basolateral membranes. We investigated both proximal tubular transport as well as biotransformation of GSH *S*-conjugates of two model compounds; firstly menadione, a quinoid-compound known to induce toxicity through redox cycling and detoxified by γGT (Brown *et al.*, 1991; Haenen *et al.*, 1994)

and secondly ethacrynic acid, an α,β-unsaturated ketone known to undergo reversible GSH conjugation (Koechel and Cafruny, 1974; Ploemen *et al.*, 1994).

MATERIALS AND METHODS

Chemicals

[^{35}S]-GSH (123.8 Ci/mmol; > 90% pure) was purchased from New England Nuclear (Boston, MA). Dithiotreitol, acivicin, L-cysteine (Cys), cysteinylglycine (CysGly) and probenecid were obtained from Sigma (St. Louis, MO, USA). NADPH and GSH reductase were from Boehringer Mannheim. Menadione (2-methyl-1,4-naphthoquinone) was obtained from Aldrich (Brussels, Belgium). GSH and *N*-acetylcysteine (*N*Ac) was purchased from Janssen (Beerse, Belgium). Nycodenz (iohexol) was from Nycomed AS (Oslo, Norway). All other chemicals were of the highest purity available.

Synthesis of [^{35}S]-MGNQ

An aqueous solution of 39.5 mM [^{35}S]-GSH (1714 dpm/nmol), 2.5 mg/ml NADPH and 0.83 U/ml GSH reductase in 281 μl of 10 mM dithiotreitol was stirred under nitrogen for 15 min. at room temperature. A solution of 2-methyl-1,4-naphthoquinone (0.13 mmol in 1.365 ml of 95% ethanol) was added dropwise. After an additional stirring for 10 min. at room temperature, the reaction mixture was stored overnight at 4°C under nitrogen. The resulting yellow solution and precipitate was collected and extracted four times with ethylacetate to remove excess 2-methyl-1,4-naphtoquinone. The reaction mixture was stored under nitrogen at 4°C.

Purification of [^{35}S]-MGNQ standard

Aliquots of the reaction mixture were subjected to preparative reversed-phase (RP)-HPLC analysis. A Zorbax ODS column (21.2 mm x 250 mm); (DuPont, Wilmington UK) fitted with a Co:Pell ODS precolumn was eluted isocratically at 4 ml/min with 65% MeOH and 35% water (pH 4 with acetic acid). UV adsorbance of eluent was monitored at 254 nm. [^{35}S]-MGNQ eluted after 14 min. and this peak was collected in 0.3 min. fractions using a Redifrac fraction collector (Pharmacia). Forty μl of each fraction was pipetted into a counting vial and after addition of 4 ml of scintillation fluid (Ultima Gold, Packard) radioactivity was counted in a liquid scintillation counter (Tricarb 1600, Packard). The fractions displaying the highest radioactivity were combined and purity was assessed by analytical HPLC analysis. Radiochemical purity of [^{35}S]-MGNQ could not exceed 72% (Fig. 4A). In this elution pattern several unidentified impurities were present (peaks 1,2,3 and 5). Specific activity of radiolabeled MGNQ was 0.16 μCi/μmol.

HPLC analysis

Reversed-phase HPLC was performed using a Perkin-Elmer Series 4 HPLC, equipped with a 250 x 4.6 mm I.D. Lichrosorb 5C18 column (Hichrom, Reading, UK) fitted with a Co:Pell ODS precolumn. The mobile phase contained 20% (v/v) MeOH and 80% of an aqueous solution of 20 mM Tris (pH 2.5; ortho phosphoric acid). A linear gradient to 100% MeOH in 20 min was applied. The flow rate was 1 ml/min and the eluent was monitored at 254 nm. The column was allowed to equilibrate for 5 min at the initial conditions between injections. MGNQ eluted after 12.5 min. Radioactivity in the eluent was determined by counting 0.5 ml fractions in a liquid scintillation counter as described. Identification of the metabolites was based on the co-eluation of radioactivity with unlabeld, authentic standards synthesized as described by Nickerson *et al.* (1963).

Synthesis of thioether conjugates of ethacrynic acid

For the synthesis of conjugates of ethacrynic acid and glutathione (EASG), cysteinylglycine (EACysGly), cysteine (EACys) and *N*-acetylcysteine (EA*N*Ac), respectively, a procedure described by Ploemen *et al.* (1990) was applied with minor modifications. A solution of 0.33 mol ethacrynic acid in 5 ml ethanol/water (1:1) was added to an equimolar solution of GSH, CysGly, Cys or *N*Ac in 5 ml ethanol/water (1:1) adjusted to pH 8-9 with saturated $NaHCO_3$ and stirred for 48h at room temperature. Ethanol was evaporated in vacuo and the aqueous phase was frozen in dry ice/acetone and lyophilized. The residue was dissolved in 2 ml of saturated $KHCO_3$ and 5-8 ml of 1% (v/v) phosphoric acid was added. To the stirring solution droplets of 10% (v/v) of phosphoric acid was added until the solution became cloudy. After centrifugation (10 min/4000 rpm), the supernatant was decanted and the pellet was dissolved in water after adjustment of the solution to pH 6 with ammonia. Purity of freeze/dried synthesis products exceeded 95%, as judged by RP-HPLC analysis. The identity of the conjugates was confirmed by field desorption mass spectrometry using a MS 902 equipped with a VG-ZAB console. The following signals were recorded (*m/z*) $(M+H)^+$: 610 (EASG), 463 (EACysGly), 424 (EACys) and 466 (EA*N*Ac). ^{1}H-NMR spectral analysis of the four thioether conjugates of EA (in D_2O) on a Bruker CXP-300 confirmed their identity. Proton signals of cys α and cys β were found in the region of δ 4.3-4.5 and 2.8-3.0, respectively. Proton signals of gly, glutamyl and *N*Ac were found in regions of δ 3.6-3.9, 2.0-2.6 and 2.0, respectively.

Synthesis of [^{14}C]-labeled EASG

The [^{14}C]-labeled glutathione conjugate of ethacrynic acid was synthesized according to the method described by Ploemen *et al.* (1994). [^{14}C]-labeled EASG was 97% pure as judged by RP-HPLC analysis. Specific activity of the conjugate was 29.28 μCi/μmol.

HPLC analysis of ethacrynic acid thioether conjugates

For chromatographic separation a modified method of Di Pietra *et al.* (1994) was used. HPLC analysis was performed on a Merck-Hitachi HPLC system fitted with a Hypersil 5C18 (200 x 3 mm I.D.) column (Chrompack, Middelburg, The Netherlands). The mobile phase consisted of 72% (v/v) MeOH and 28% of an aqueous solution of triethylammonium phosphate (TEA: 0.05 M, pH 3.0 with ortho-phosphoric acid). The column was eluted with 28:72 (MeOH/TEA) for 20 min. Then, a linear gradient to 95% methanol in 30 min was applied. Flow rate was 0.8 ml/min and the eluent was monitored at 270 nm. Between injections, the column was allowed to equilibrate for 5 min at the intial conditions. The following k' values were obtained: EACysGly (16), EACys (17), EASG (18), EA*N*Ac (20) and EA (23). For chromatographic analysis of radioactive samples, aliquots were injected on the column together with a mix of unlabeled, authentic standards. Simultaneously with HPLC analysis, radioactivity was measured using an on-line radiochemical detector (Radiomatic Flo-one/beta, Canberra Packard, The Netherlands).

Cell isolation and culture

Female Wistar rats (Harlan CPB, Zeist) weighing 180-230 g were used for isolation of RPT cells according to a method by Boogaard *et al.* (1989). The proximal tubular origin of isolated cells was confirmed by staining monolayers of these cells for γGT according to a method by Rutenburg *et al.* (1969). Primary RPT cells were seeded on collagen-coated (Transwell) tissue culture inserts (Costar, Badhoevedorp, The Netherlands) at a density of 2.10^5 cells/cm^2 (Fig. 1). The cells were cultured at 37°C in a humified atmosphere containing 5% CO_2 in Williams' E (WE) medium (Flow Irvine, Scotland) supplemented with 10% foetal calf serum (Gibco, Glasgow, Scotland) and gentamycin (50 μg/ml) (Gibco, Scotland). The cell cultures were left undisturbed in a humified incubator for 4 days in order to grow to confluency. Confluency was checked by measurement of the Trans Epithelial Electrical Resistance (TEER) across the monolayer as described by Haenen *et al.* (1994).

Transport experiments

Confluent monolayers were washed with Hanks' Balanced Salt Solution (HBSS) without phenol red. The apical (A) and basolateral (B) chambers were filled with 0.5 and 1.5 ml of HBSS, respectively, containing 10 mM $NaHCO_3$ and 20 mM HEPES (pH 7.4). For A⇒B and B⇒A transport of [^{35}S]-MGNQ or [^{14}C]-EASG, 125 μM [^{35}S]-MGNQ or 14 μM [^{14}C]-EASG was added to the donor compartment. Tight junctional integrity during the experiments was assessed by monitoring leakage of [^{3}H]-mannitol across the monolayer. To rule out interference between mannitol and MGNQ or EASG, [^{3}H]-mannitol (13 nM) was added to the opposite compartment. At indicated time points, 60 and 180 μl samples were drawn from the apical and basolateral chambers, respectively. Twenty five % of each sample was mixed with 4.5 ml of scintillation

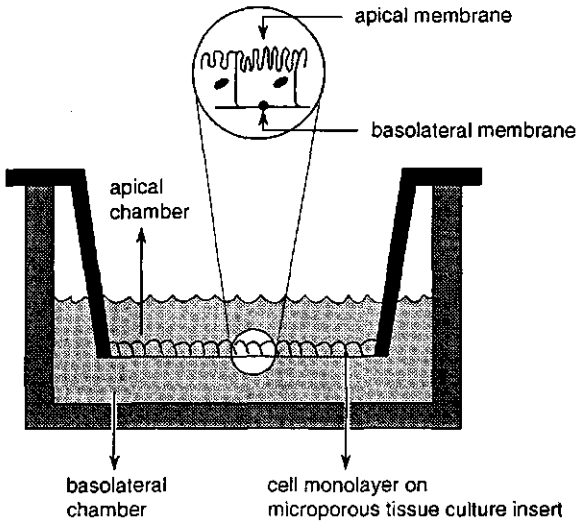

Figure 1. Cross-section of a Transwell tissue culture insert. Renal proximal tubular cells attach to the microporous insert with their basolateral membrane and a differentiated, confluent monolayer develops. As a result, the medium compartment is divided into an apical (A) and a basolateral (B) chamber which enables A⇒B and B⇒A transport across the monolayer (Mertens et al., 1988; Haenen et al., 1994).

fluid and radioactivity was counted in a liquid scintillation counter equipped with a dual label program for ^{3}H and ^{35}S or ^{14}C. The rest of the aliquots were stored at -20°C until HPLC analysis. At the end of the transport experiments, Transwell inserts were washed three times in ice-cold Phoshate Buffered Saline (PBS) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and with ice-cold PBS alone. As a result, opening of tight junctions occurred as a result of EDTA-mediated Ca^{++} withdrawal and radioactivity trapped in the paracellular space was eliminated. In addition, cell-associated radioactivity was corrected for cellular adherence. The monolayers were cut out of the insert and sonicated in 0.5 ml of 0.5% (v/v) Triton X-100 for 10 min. After centrifugation (9000 rpm, 10 min), the supernatant was pipetted into a counting vial and radioactivity was counted as described. Transepithelial passage of ^{35}S and ^{14}C-radioactivity comprises also paracellular leakage of both radiolabels. After correction for paracellular leakage of ^{35}S and ^{14}C-radioactivity by measuring [^{3}H]-mannitol leakage across the monolayer, net transport of ^{35}S and ^{14}C-radioactivity was determined.

Metabolism of [^{14}C]-EASG by freshly isolated RPT cells

Freshly isolated RPT cells (2.10^5) were seeded in a 12-well tissue culture plate containing 1.0 ml HBSS without phenol red containing 10 mM $NaHCO_3$ and 20 mM HEPES (pH 7.4). 14 μM [^{14}C]-EASG was added and metabolism experiments were monitored for 8h under culture conditions. At indicated time points aliquots of the medium were collected and stored at -20°C until HPLC analysis.

Stability experiments

Stability of EASG, EACysGly and EACys was evaluated by dissolving 500 μg of conjugate in 1 ml HBSS without phenol red and incubating these solutions for 8h under culture conditions. At indicated time points 50 μl samples were drawn and subjected to HPLC analysis as described. The amount of conjugate present in the incubation was expressed as area under the curve in arbitrairy units after integration of the peaks.

Statistics

When appropriate, data are expressed as means ($\pm$ SEM) of at least three experiments in triplicate. Statistical comparisons were made between groups using the Student's *t*-test (one tailed). Significance was accepted at $p \leq 0.05$.

RESULTS

Junctional integrity of monolayers

It was demonstrated previously that RPT cells cultured on porous supports of collagen coated tissue culture inserts reach confluency after 4 days of culture (Haenen *et al.*, 1994). Monitoring the development of tight junctions by measuring the TEER revealed that confluent monolayers displayed a net TEER of at least 400 ohm/cm^2 (Haenen *et al.*, 1994). In addition, monolayer confluency can also be monitored by assessing the leakage of a marker of passive, paracellular diffusion like mannitol. Fig. 2 shows the A⇒B and B⇒A paracellular leakage of [^{3}H]-mannitol across confluent RPT cell monolayers monitored for 8h. A⇒B leakage of [^{3}H]-mannitol was significantly higher than B⇒A leakage. Linear regression analysis revealed that A⇒B and B⇒A leakage of [^{3}H]-mannitol proceeded at a rate of 1.5% and 1.1%/cm^2/h, respectively.

Transepithelial transport of [^{35}S]-MGNQ

Fig. 3 shows the transepithelial passage of [^{35}S]-MGNQ and [^{3}H]-mannitol across RPT cell

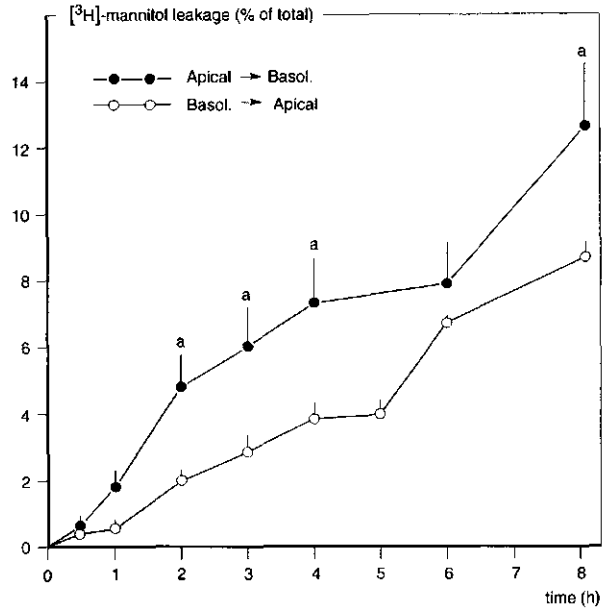

Figure 2. *Transepithelial passage of [^{3}H]-mannitol across confluent RPT cell monolayers. [^{3}H]-mannitol was added to either the upper or the lower compartment of the culture insert at 13 nM. After different times of incubation, samples were taken from both the donor and the opposite compartment as described in the Materials and Methods section. Transepithelial passage of [^{3}H]-mannitol is expressed as % of total radioactivity in the donor compartment. Results presented are the mean (± SEM) of at least 8 experiments. At 2, 3, 4 and 8h, A⇒B transport of mannitol was significantly different from B⇒A transport ($p \leq 0.001$).*

monolayers. A⇒B and B⇒A passage of [^{35}S]-MGNQ did not differ from paracellular leakage of [^{3}H]-mannitol. To discriminate between passage of MGNQ and a hydrolysis product thereof, A⇒B passage of [^{35}S]-MGNQ across RPT cell monolayers was also studied in the presence of acivicin, an inhibitor of γGT. At the end of the experiment (8 h), acivicin significantly reduced A⇒B transepithelial passage of [^{35}S]-MGNQ. To find out whether the GSH conjugate of menadione was basolaterally taken up by the RPT cells via a probenecid-inhibitable GSH transporter, we studied B⇒A passage of [^{35}S]-MGNQ in the presence of probenecid. Probenecid did not have an effect on B⇒A passage of [^{35}S]-MGNQ. In these experiments, no A⇒B or B⇒A transepithelial transport of MGNQ could be observed.

After 8h of incubation of [^{35}S]-MGNQ in the absence of RPT cells spontaneous breakdown

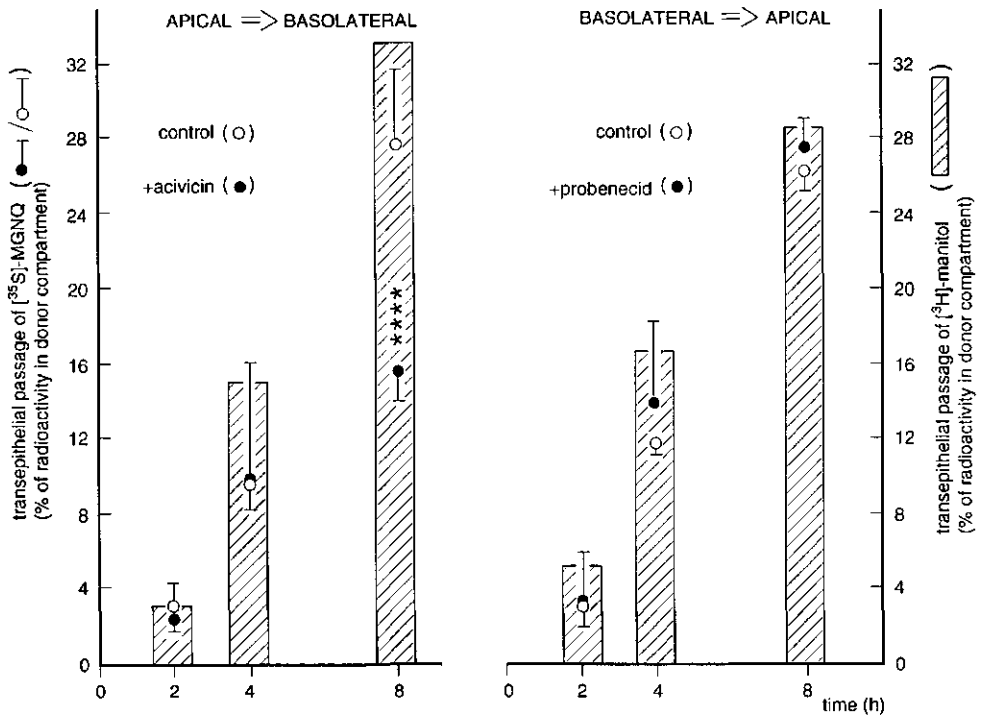

Figure 3. *Transepithelial passage of total [^{35}S]-radioactivity and [^{3}H]-mannitol across RPT cell monolayers. Transepithelial passage of radioactivity is expressed as % of radioactivity in the donor compartment. (**** indicates significantly different ($p \leq 0.001$) from control A⇒B transport of [^{35}S]-MGNQ after 8h).*

occurred and an increase in concentration of peaks 1,2,3 and 5 had taken place (Fig. 4D). After 4h of incubation of [^{35}S]-MGNQ with γGT, the parent compound had been metabolised into a metabolite which eluted after 16 min (peak 7) (Fig. 4B). After metabolism of [^{35}S]-MGNQ by RPT cell monolayers, a metabolite with an identical retention time was observed in the apical medium (Fig. 4C). No such metabolite was detected after metabolism of MGNQ in the presence of acivicin (Fig. 4E). The metabolite was isolated from the γGT incubations and its identity was assessed by nuclear magnetic resonance analysis and field desorption mass spectral analysis. The ^{1}H-NMR spectrum shows a singlet at δ 2.38 ppm (3H) which is assigned to the CH_3 group at position 10, a singlet at δ 3.63 ppm (2H) of the CH_2 group of glycine, a singlet at δ ppm 4.11 (2H) of a CH_2 group of the cysteinylglycine moiety. In addition, the spectrum exhibits a multiplet

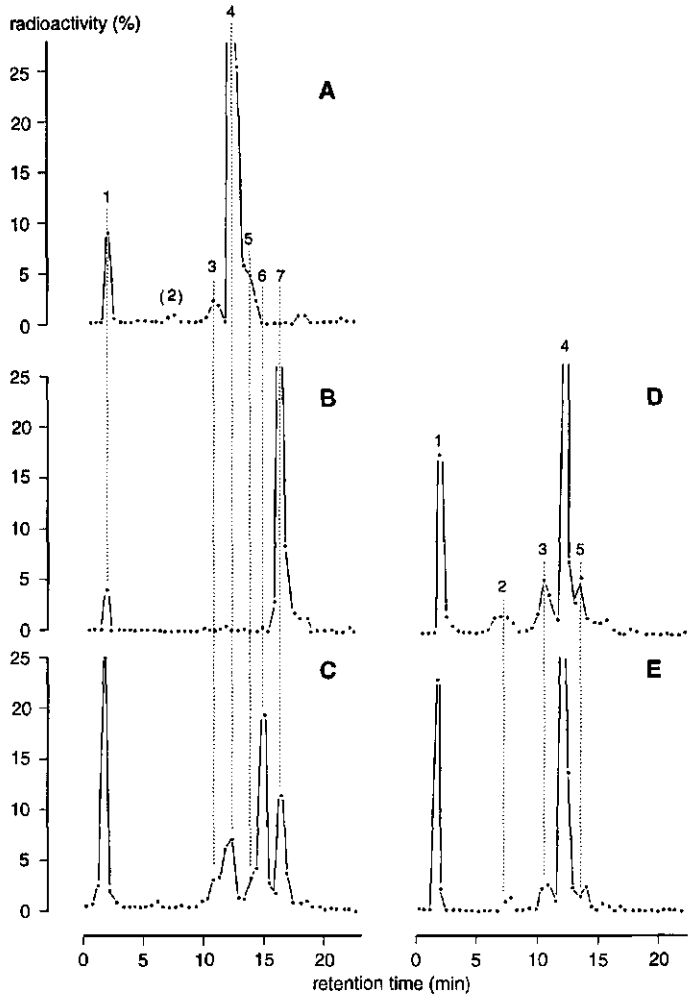

Figure 4. *HPLC elution patterns of [^{35}S]-MGNQ at the onset of incubation (A); after incubation with 150 mU γGT for 8h (B); after apical incubation for 8h with an RPT cell monolayer (C); after 8h of incubation in the absence of RPT cells (D) and after apical incubation for 8h with a RPT cell monolayer in the presence of 500 μM acivicin (E). Analysis was performed using a Lichrosorb 5RP18 column (150 x 4.6 mm I.D.). Amount of radioactivity eluted from the column is expressed as % of total radioactivity in the peaks. Peak 1, 2, 3 and 5 represent unidentified impurities due to spontaneous breakdown of MGNQ. Peak 6 presumably is 1,4-naphthothiazine. Peak 4 corresponds to the parent compound, [^{35}S]-MGNQ. Peak 7 corresponds to the 1,4-naphthothiazine adduct of the cysteinylglycine conjugate of menadione.*

at δ ppm 7.50 (2H), a double doublet at δ ppm 8.15 (1H) and a double doublet at δ ppm 8.49 (1H), all from the aromatic protons. Using mass spectral analysis a signal at *m/e* 330 was observed which corresponds to the overall structural formula of the 1,4-naphthothiazine metabolite formed from the cysteinylglycine conjugate of menadione. Presumably, peak 6 is 1,4-naphthothiazine formed through the action of dipeptidases.

Transepithelial transport of total [^{14}C]-radioactivity and [^{3}H]-mannitol

A⇒B passage of [^{14}C]-EASG and B⇒A passage of [^{3}H]-mannitol increased linearly with time (Fig. 5). A⇒B passage rate of [^{14}C]-EASG was 4.2%/h/cm^2 whereas B⇒A passage of [^{3}H]-mannitol occurred at a much slower rate of 1.2%/h/cm^2. A⇒B passage of [^{14}C]-EASG reached a maximum value of 33.9 $\pm$ 1.8% at 8h of incubation. At this time point, B⇒A passage of [^{3}H]-mannitol reached a maximum value of 9.9 $\pm$ 1.6%. As a result, A⇒B transport of [^{14}C]-EASG was 24% after 8h of incubation. In the presence of acivicin, A⇒B passage of [^{14}C]-EASG occurred at a slower rate (2.2%/h/cm^2) than A⇒B passage of [^{14}C]-EASG. At 8h of incubation, this passage reached a maximum value of 19.9 $\pm$ 4.8% which is significantly ($p \leq 0.001$) different from A⇒B passage of [^{14}C]-EASG in the absence of acivicin. As a result, the amount of [^{14}C]-EASG transported was 10% at this time point.

In comparison with A⇒B passage, B⇒A passage of [^{14}C]-EASG was very low and increased proportionally with time (1.0%/h/cm^2). Surprisingly, A⇒B passage of [^{3}H]-mannitol occurred at a much higher rate (4.6%/h/cm^2) reaching a maximum value of 38.9 $\pm$ 1.1% after 8h of incubation. No B⇒A transport of [^{14}C]-EASG was observed across the RPT cell monolayer.

Metabolism and transport of [^{14}C]-EASG

Apical exposure of RPT cell monolayers to [^{14}C]-EASG resulted in the disappearance of this conjugate and the concomitant formation of [^{14}C]-EA in this compartment of the tissue insert (Fig. 6). No other thioether conjugates of EA were identified in the media. At 8h of incubation, only 1.7% of [^{14}C]-radioactivity is present as EASG whereas 76% of [^{14}C]-radioactivity is present as EA. After 4 h of A⇒B passage of [^{14}C]-EASG, 4.7% of radiolabel was recovered in the basolateral compartment as EA. At this time point, no EASG was detected in this compartment. However, after 8h of incubation 15.5% of radiolabel was identified as EASG, whereas 4.4% was present as EA in the basolateral compartment.

To establish the role of γGT in the metabolism of EASG, RPT cell monolayers were apically pre-incubated with acivicin. In the presence of acivicin, apical metabolism of [^{14}C]-EASG was inhibited and at 8h of incubation 94.4% of total radiolabel was present as EASG whereas only 3.2% of radiolabel was recovered as EA. In this incubation, only EASG was detected in the basolateral compartment. At 8h of incubation, passage of [^{14}C]-EASG to this compartment was 10.7%.

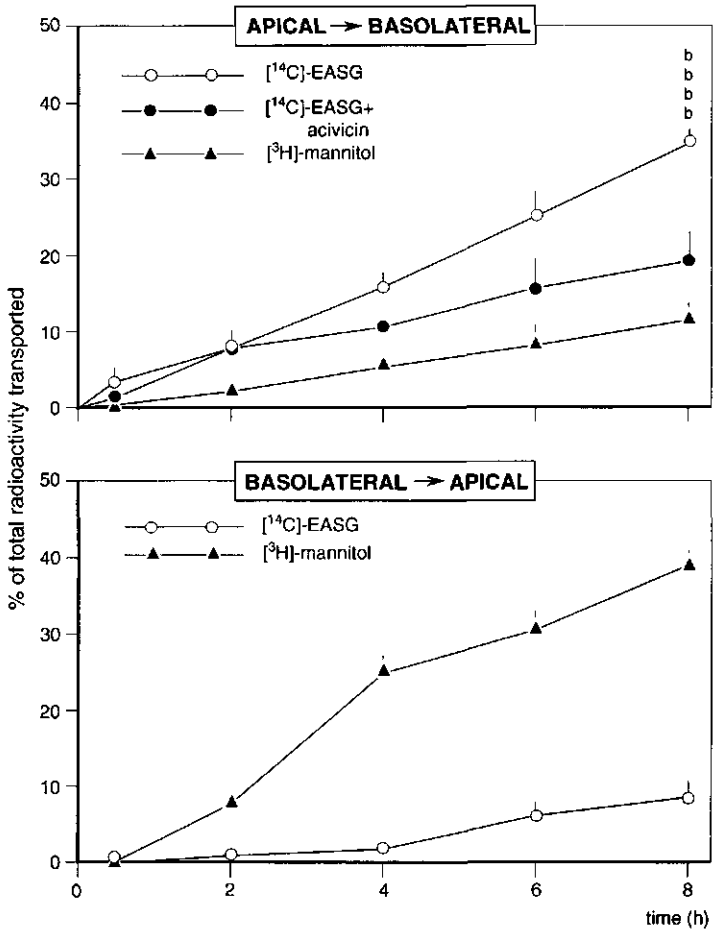

Figure 5. Transepithelial transport of total [^{14}C]-radioactivity and [^{3}H]-mannitol across RPT cell monolayers. In control transport incubations, both transport of [^{14}C] and [^{3}H]-radioactivity was monitored. To prevent interference between mannitol and EASG, transport of [^{3}H]-mannitol and [^{14}C]-EASG occurred in opposite directions. Results are the means (± SEM) of 4 incubations and are presented as % of total radiolabel in the donor compartment.

Basolateral exposure of RPT cell monolayers to [^{14}C]-EASG in the absence or presence of probenecid showed that EASG was metabolized at a much slower rate than at the apical side of the monolayer. After 8h of incubation 66% and 78%, respectively, of [^{14}C]-radioactivity was identified as EASG, whereas the rest of radiolabel was recovered as EA. Also in these

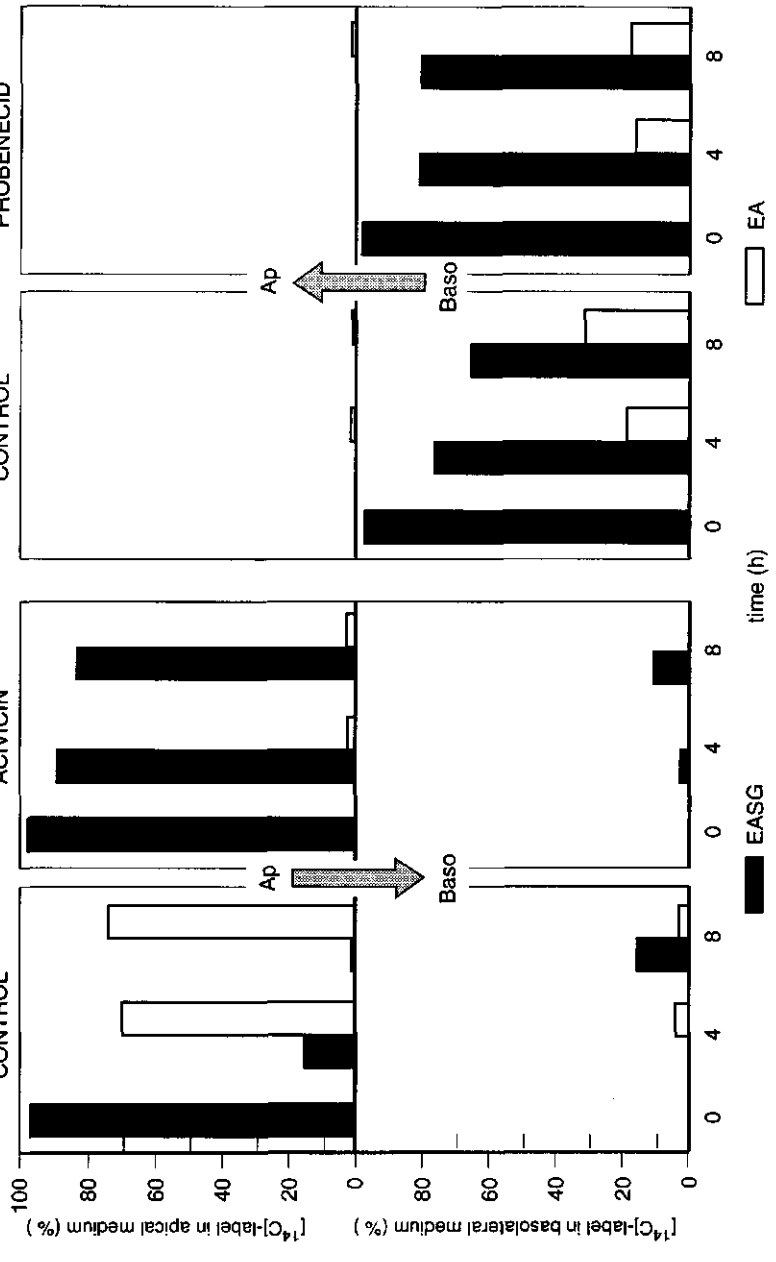

Figure 6. Percentage of total [^{14}C]-radioactivity present as EASG and EA in apical and basolateral media after apical (A: left panel) or basolateral (B: right panel) exposure of RPT cell monolayers to [^{14}C]-EASG. Confluent monolayers of RPT cells were exposed to 14 μM [^{14}C]-EASG. When indicated, monolayers were pre-incubated for 30 min on the apical side with 500 μM acivicin or on the basolateral side with 500 μM probenecid. After this period, RPT cell monolayers were simultaneously exposed to acivicin or probenecid in the presence of [^{14}C]-EASG for 8h. Results are the fraction of the added [^{14}C]-EASG recovered in the apical or basolateral compartments as EASG or EA at the indicated times. The results are from one typical experiment out of four.

incubations, no other thioether conjugates of EA were identified. Apart from a very small amount of EA (1%), no conjugates were recovered in the apical compartment.

After the transport experiments, the cell-associated [^{14}C]-radioactivity was determined (expressed as mean ± SEM; n=4). Total [^{14}C]-radioactivity in monolayers after A⇒B passage was 205 ± 30 dpm. A significant reduction in cell-associated radioactivity ($p \leq 0.001$) was observed in monolayers treated with acivicin: 38 ± 11 dpm. After B⇒A passage of [^{14}C]-EASG, cell-associated [^{14}C]-radioactivity was 365 ± 58 dpm, which was significantly different ($p \leq 0.05$) from the amount of radioactivity in monolayers after A⇒B passage.

Metabolism profile of [^{14}C]-EASG by RPT cells

Fig. 7 shows a metabolism profile after apical metabolism of EASG by a RPT cell monolayer (left panel). Even at 0.5, 1 and 2h after the addition of EASG, only EASG and EA could be detected in the media. To find out whether cultured RPT cells have lost the capacity to metabolize EASG into EACysGly, EACys or EA*N*Ac, we monitored the metabolism of EASG by

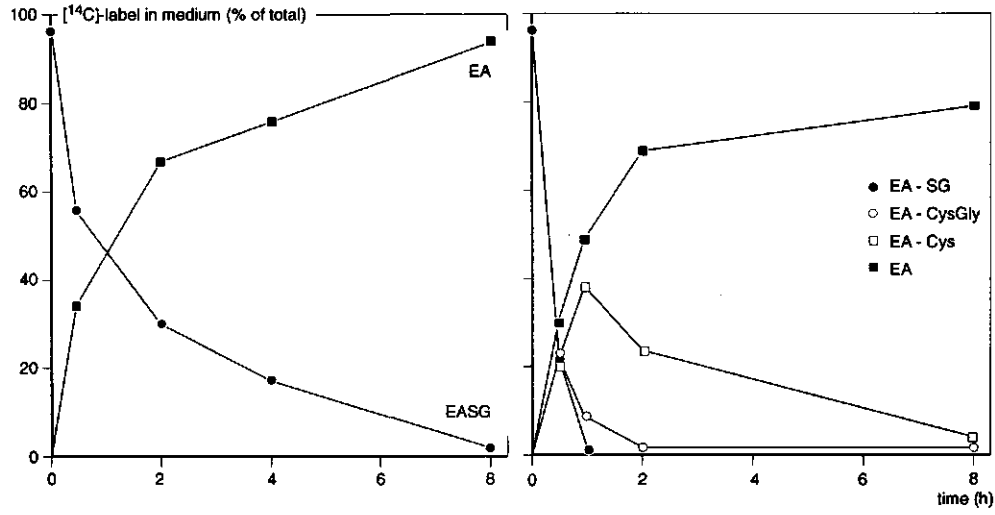

Figure 7. *Metabolism profile of [^{14}C]-EASG by RPT cell monolayers (left panel) and freshly isolated RPT cells (right panel). Freshly isolated RPT cells (2.10^5) were incubated in a 24-wells plate as described in the Materials and Methods section. Results are presented as % of radiolabel recovered as EA or thioether conjugates thereof in the medium. Results of a typical experiment are shown.*

freshly isolated RPT cells. Since confluent RPT cell monolayers contain approximately 2.10^5 cells after 4 days of culture, we incubated an equal amount of freshly isolated RPT cells with 14 μM [^{14}C]-EASG for 8h. The right panel of Fig. 7 shows a metabolism profile of EASG by freshly isolated RPT cells. Probably as a result of higher γGT and dipeptidase activities in these cells compared to monolayers of RPT cells, EACysGly and EACys were identified in the media in addition to EASG and EA. EACysGly and EACys reached a maximum concentration after 0.5 and 1h of metabolism, respectively. At these time points, respectively, 21% and 38% of [^{14}C]-label was present as these conjugates. Also in the presence of freshly isolated RPT cells, no mercapturic acid of EA was detected in the media.

In addition, EASG is degraded more quickly by freshly isolated cells than by RPT cell monolayers. After 0.5h of metabolism, freshly isolated cells reduced the amount of EASG to 21%, whereas 55% of EASG was left after γGT-mediated metabolism of EASG in the monolayer at this time point.

Stability of thioether conjugates of EA

To find out whether reversible conjugation of EA with thioethers plays a role in the metabolism of EASG by RPT cells, we investigated the stability of EASG, EACysGly and EACys in phosphate buffer (pH 7.4). Fig. 8 shows that EASG is stable for 8h at physiological pH. However, both EACysGly and EACys are instable at this pH value. The amount of EACysGly was reduced to 50% within 1h of incubation. After 3h, no EACys could be detected in the medium.

DISCUSSION

In order to study transepithelial transport and metabolism of GSH conjugates by RPT cells, we cultured these cells to confluency on microporous filters. This allows selective exposure at one site, and measurement of eventual transport at the other side. As judged by leakage of [^{3}H]-mannitol, monolayers of RPT cells reached confluency after 4 days of culture. In our system mannitol leakage was 10-fold lower compared to confluent monolayers of LLCPK$_1$ cells (Fouda *et al.*, 1989) which illustrates the tight junctional integrity of the presently used cell monolayer.

Since 50-60% of circulating GSH is supposed to be removed via basolateral uptake mechanisms (Lash and Jones, 1984), one might expect that a considerable amount of GSH conjugates could also be excreted via these mechanisms. However, no specific B⇒A transport of both GSH conjugates across RPT cell monolayers was observed. Similarly, Rabito (1986) and

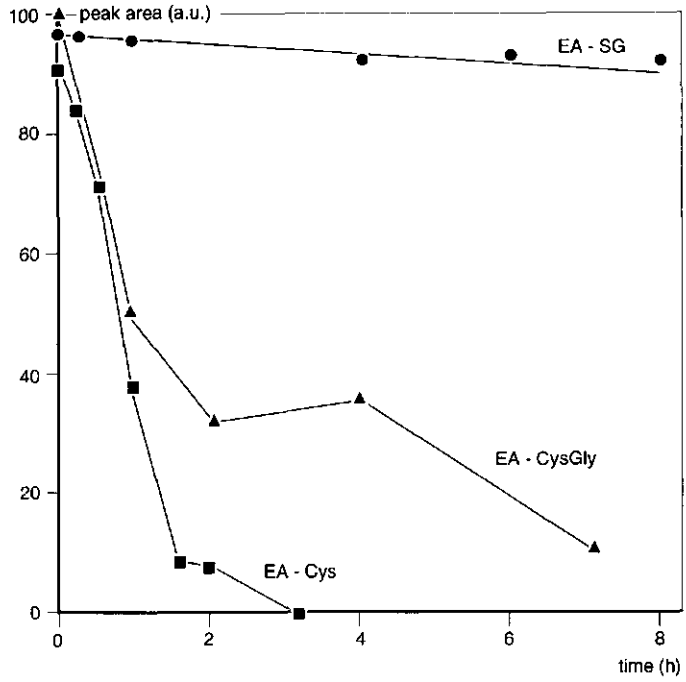

Figure 8. *Stability of thioether conjugates of EA in phosphate buffer (pH 7.4). EASG, EACysGly and EACys were incubated as described in the Materials and Methods section. Results are expressed as area under the curve in arbitrary units. A typical experiment is shown.*

Mertens *et al.* (1988) could also not detect B⇒A transport of para-aminohippuric (PAH) acid across monolayers of $LLCPK_1$ cells. It seems therefore that, perhaps as a result of dedifferentiation, both $LLCPK_1$ and RPT cells lack functional basolateral transport systems.

At long (8h) exposure times, for [^{35}S]-MGNQ a measureable amount of total [^{35}S]-radioactivity does pass the monolayer. However, this is presumably the result of a decline in monolayer integrity. Quinones are known to undergo extensive redox cycling in an atmosphere of 20% oxygen as used here and this will result in toxicity (Haenen *et al.*, 1996).

Since transepithelial passage of both [^{35}S]-radioactivity and [^{3}H]-mannitol were comparable, A⇒B passage of [^{35}S]-radioactivity is likely to occur solely via paracellular leakage. However, this paracellular leakage may mask a transcellular component in A⇒B passage of total [^{35}S]-radioactivity. The observation that acivicin, an inhibitor of γGT, significantly reduced A⇒B passage of total [^{35}S]-radioactivity after 8h, suggests that a product of the hydrolysis of the GSH

conjugate by this enzyme is transported transcellularly. To investigate this possibility, the metabolism of [^{35}S]-MGNQ was studied.

Cysteinylglycine and cysteine conjugates of both naphtho -and benzoquinones are known to undergo an intramolecular cyclization reaction yielding 1,4-naphthothiazines (Kuhn and Hammer, 1951; Monks *et al.*, 1990). Indeed, in the RPT cell culture system MGNQ is metabolised into two cyclization products. The first product was definitively identified as 2*H*-(3-glycinyl)-9-hydroxy-10-methyl-1,4-naphthothiazine, the cyclized cysteinylglycine conjugate of menadione. The second product was presumably 1,4-naphthothiazine formed by loss of glycine from the first product via the action of dipeptidases. No other metabolites were detected in the media. Because quinone-thioethers are metabolized *in vivo* mainly into mercapturic acids and to a much lesser extent into cyclization products (Rivera *et al.*, 1994), *in vitro* metabolism of quinone-thioethers therefore does not completely reflect metabolism *in vivo*, since no *N*-acetylation reaction occurs as was also found for the GSH conjugate of 2,5-difluoronitrobenzene (Haenen *et al.*, 1995).

Net transport of total [^{14}C]-radioactivity could be observed from A⇒B direction. The compounds detected at the basolateral side were EASG and free EA only. To understand this phenomenon, the stability of the various conjugates was studied. Whereas EASG was stable at pH 7.4 for up to 8h, both EACysGly and EACys deconjugated with a half life of approximately 1h at pH 7.4, leading to relatively large amounts of free, unconjugated EA. In agreement with this, inhibition of γGT with acivicin significantly reduced A⇒B transport of [^{14}C]-radioactivity and inhibited the formation of free EA. In other words, not the GSH conjugate is transported from A⇒B direction but rather the cysteine adduct and/or free EA. The GSH conjugate observed would then be the result of renewed conjugation of the free EA in the RPT cell followed by excretion of the GSH conjugate at the basolateral side.

From both conjugates studied, it has become clear that the confluent monolayers of RPT cells do not or no longer possess active basolateral transport systems for GSH conjugates. However, they are still useful for studying biotransformation reactions of thioether conjugates.

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CHAPTER 5

In vitro metabolism of 5-fluoro-2-(glutathionyl)-nitrobenzene by kidney proximal tubular cells studied by ^{19}F-NMR

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SUMMARY

Proximal tubular biotransformation of the glutathionyl (GSH) conjugate derived from 2,5-difluoronitrobenzene (5-fluoro-2-glutathionyl-nitrobenzene) was studied by means of ^{19}F-NMR. This method allows a direct and specific detection of the fluorinated metabolites formed, at a detection limit of 1 μM for an overnight NMR run. Incubation of a monolayer of LLCPK$_1$ cells with 100 μM 5-fluoro-2-glutathionyl-nitrobenzene (F*G*NB) for 24h showed that these cells metabolize this GSH conjugate into the corresponding cysteinylglycyl (F*CysGly*NB) and cysteine (F*Cys*NB) conjugate. The expected *N*-acetylcysteine conjugate (F*N*AcNB) however was not formed as an endproduct. Additional experiments demonstrated the absence of *N*-acetyltransferase activity in LLCPK$_1$ cell lysates incubated with F*Cys*NB and also the rapid loss of this activity in isolated renal proximal tubular (RPT) cells: freshly isolated RPT cells do convert F*Cys*NB to F*N*AcNB as major metabolite but, upon cultivation, quickly lose this capacity. Since uptake of F*Cys*NB might also be a limiting factor, transport of F*Cys*NB from the apical to the basolateral side of the cultured RPT cells was investigated. No indication for such transport was obtained. Thus, the absence of mercapturic acid formation in LLCPK$_1$ cells and cultured RPT cells is the result of a decline in *N*-acetyltransferase activity and perhaps a deficient cellular uptake of the cysteine conjugate.

INTRODUCTION

Halogenated nitrobenzenes are used as intermediates in the manufacture of dyes, pharmaceuticals and agrochemicals.

Metabolism of nitrobenzenes has been investigated in rabbits (Bray *et al.*, 1956, 1958) in rats (Levin and Dent, 1982; Rickert, 1987; Rickert and Held, 1990; Yoshida *et al.*, 1991) and in humans (Piotrowski, 1967; Yoshida *et al.*, 1992).

Until now, limited information exists concerning the renal contribution in the metabolism and excretion of nitrobenzenes. The observation of mercapturic acids in the urine of rabbit, rat, dog, human and other mammals exposed to aromatic nitrocompounds (Bray *et al.*, 1956; Rickert, 1987; Yoshida *et al.*, 1992) suggests a substantial role for the mercapturic acid pathway. This implies that renal excretion of glutathionyl conjugates involves proximal tubular processing of these conjugates via cysteinylglycine and cysteine conjugates into their *N*-acetylcysteine derivatives (Monks and Lau, 1987). To study these events mechanistically, $LLCPK_1$ and RPT cells were used. $LLCPK_1$ is one of the best characterised cell lines of proximal tubular origin. Both $LLCPK_1$ and RPT cells form confluent monolayers of partly differentiated cells on porous supports of tissue culture inserts (Handler, 1983; Mertens *et al.*, 1988; Boogaard *et al.*, 1989). In addition, these experimental setups have been proven to be successful when studying proximal tubular transport of organic cations (Fouda *et al.*, 1990), hydrophobic drugs (Horio *et al.*, 1989) and glutathione (Scott *et al.*, 1993).

In the present paper we report on the development of a method to detect metabolites of proximal tubular biotransformation of a model halogenated nitrobenzene compound. The glutathione conjugate of a fluorinated nitrobenzene (5-fluoro-2-glutathionyl-nitrobenzene) was used as the model compound, that was metabolised by $LLCPK_1$ cells and rat RPT cells. Biotransformation of this GSH conjugate was qualitatively and quantitatively studied by means of ^{19}F-NMR.

MATERIALS AND METHODS

Chemicals

2,5-Difluoronitrobenzene, GSH and *N*-acetyl-L-cysteine were purchased from Janssen (Beerse, Belgium). Aminooxyacetic acid was from Merck (Darmstadt, Germany). AT-125 (acivicin), cysteinylglycine and cysteine were bought from Sigma (St. Louis, MO, USA). NADH was obtained from Boehringer (Mannheim, Germany). All other reagents were of the highest grade commercially available.

Conjugate synthesis

5-Fluoro-2-(glutathionyl)-nitrobenzene (FGNB), 5-fluoro-2-(cysteinylglycyl)-nitrobenzene

(F*CysGly*NB), 5-fluoro-2-(cysteinyl)-nitrobenzene (F*Cys*NB) and 5-fluoro-2-(*N*-acetylcysteinyl)-nitrobenzene (F*N*AcNB) were synthesized from 2,5-difluoronitrobenzene (2,5-DFNB) as follows. To 0.3 g (1.9 mmol) of 2,5-DFNB dissolved in 2.5 ml of methanol, 2.5 ml of a solution containing 0.1 g (1.9 mmol) of sodium methanolate and 0.5 mmol reduced glutathione (GSH), cysteinylglycine (CysGly), cysteine (Cys), or *N*-acetyl-L-cysteine (*N*Ac) dissolved in methanol were added over a period of 30 min. The mixture was stirred at room temperature overnight. Upon cooling to 4°C and centrifugation (10 min at 13000 rpm) in an Eppendorf centrifuge, the supernatant was mixed with one volume of demineralized water and one volume of 0.2 M potassium phosphate pH 7.6. After another centrifugation step, resulting in a 2,5-DFNB containing pellet, the supernatant was washed three times with diethylether to remove the final 2,5-DFNB. The aqueous phase was evaporated under vacuum and the residue, containing the conjugate, was dissolved in demineralized water and analyzed by ^{19}F-NMR in 0.1 M potassium phosphate pH 7.6, and by ^{1}H-NMR, upon lyophilisation and dissolving in $^{2}H_2O$.

^{19}F-NMR analysis

^{19}F-NMR measurements were performed on a Bruker AMX 300 spectrometer as described before (Vervoort *et al.*, 1990; Rietjens and Vervoort, 1989). Between 1500 and 50000 scans were recorded, depending on the concentrations of the fluorine containing compounds and the signal to noise ratio required. The sample volume was 1.71 ml including 100 μl $^{2}H_2O$ for locking the magnetic field and 10 μl of a solution (8.4 mM) of the internal standard 4-fluorobenzoic acid (pH 7.5). Chemical shifts are reported relative to $CFCl_3$. Concentrations of the various metabolites could be calculated by comparison of the integrals of the ^{19}F-NMR resonances of the metabolites to the integral of the ^{19}F-NMR resonance of 4-fluorobenzoic acid.

^{1}H-NMR analysis

^{1}H-NMR measurements were performed on an Bruker AMX 500 spectrometer using a narrow bore 5 mm Bruker ^{1}H observe. Upon lyophilisation, the synthesized compounds were dissolved in $^{2}H_2O$ to give a final sample volume of 0.5 ml. Spectra were recorded using 60° pulses (6μs), a 10 kHz spectral width, a repetition time of 1.9 s, quadrature phase detection and quadrature phase cycling (CYCLOPS). About 150 scans were recorded. ^{1}H chemical shift values are presented relative to 3-trimethylsilyl(2,2,3,3-$^{2}H_4$)propionate.

Cell strain and culture

Epithelial pig kidney cells ($LLCPK_1$) (Flow, Scotland) at passage 190-220, were grown in tissue culture flasks (Costar, Badhoevedorp, The Netherlands) at 37°C in a humidified atmosphere containing 5% CO_2. The medium used was Williams' E (WE) (Flow, Scotland) supplemented with 10% fetal calf serum (Gibco, Glasgow, Scotland) and a mixture of penicillin (50 IU/ml) and

streptomycin (100 mg/l) (Gist-Brocades, Delft, The Netherlands). Stock cultures, seeded at 0.8-1.0 x 10^4 cells/cm^2, were subcultured every 3 to 4 days by trypsinization with 0.25% trypsin (Difco, Detroit, USA) and 0.05% EDTA (Merck, Darmstadt, Germany) after rinsing with Hanks' Balanced Salt Solution (HBSS).

Cell isolation and culture

Rat RPT cells were isolated according to a method described by Boogaard *et al.* (1989). RPT cells were seeded in tissue culture flasks and in collagen-coated transwell cell culture chambers at 2.10^5 cells/cm^2. Culture conditions were the same as for $LLCPK_1$ cells, except for the fact that gentamycin (50 μg/ml) instead of penicillin/streptomycin was used as antibiotic.

Cytotoxicity assay

To find a non-toxic concentration of F*G*NB to study metabolism by $LLCPK_1$ and RPT cells we studied cellular toxicity of F*G*NB. To this end, $LLCPK_1$ and RPT cells were seeded in 24-well tissue culture plates (Costar, The Netherlands) at a concentration of 0.8 x 10^5 cells/well. After 3 days of culture a confluent monolayer was formed. After rinsing with HBSS the cells were exposed to F*G*NB (25-400 μM) in WE medium without serum. After 24h, cytotoxicity was determined by means of lactate dehydrogenase (LDH)-leakage (Mitchell *et al.*, 1980).

In vitro metabolism studies

Renal proximal tubular cells

Freshly isolated RPT cells and confluent monolayers of RPT and $LLCPK_1$ cells (approximately 6 - 8.10^6 cells) were exposed to a non-toxic concentration of F*G*NB in WE medium without serum for 24h in culture flasks. Before the metabolism studies started, cells were rinsed with HBSS. At indicated time points samples (2 ml) were drawn from the medium. When mentioned, monolayers were first pre-incubated with 500 μM acivicin or aminooxyacetic acid (AOOA) for 15 min and thereafter incubated in the presence of F*G*NB. At the end of the experiments with $LLCPK_1$ cells, thioether conjugate metabolism was also examined intracellularly. To this end, cells were trypsinized as described above, resuspended in WE medium and disrupted via three freeze/thaw sessions. The cell homogenate was centrifuged at 9000 *g* for 10 min. The supernatant (S9-mix) was decanted and stored, together with the medium samples, at -20°C until ^{19}F-NMR analysis.

Cell lysates

In addition, F*G*NB metabolism was also studied with cell lysates of (6 - 8.10^6) $LLCPK_1$ cells. After the freeze/thaw sessions, cell lysates were incubated with a non-toxic concentration of F*Cys*NB for 24h under culture conditions. Upon centrifugation (10 min at 9000 *g*) the resulting

supernatant was used for ^{19}F-NMR analysis.

N-acetylation capacity

Freshly isolated RPT cells (8.10^5 cells) were seeded into 25 cm^2 tissue culture flasks and cultured as described. Immediately after seeding, and after 1, 2 and 3 days of culture, cells were exposed to a non-toxic concentration of FGNB dissolved in WE medium without serum for 24h. Medium samples were stored at -20°C until ^{19}F-NMR analysis.

Transport of FCysNB by RPT cells

To measure transport of FCysNB by RPT cell monolayers, confluent monolayers on porous supports were apically incubated with 100 μM FCysNB for 24h. At 2, 4, 8, and 24h medium samples of the opposite (basolateral) chamber were drawn and analysed for fluorinated compounds.

RESULTS

Spectral characteristics of reference compounds

Table 1 presents the ^{19}F-NMR and ^{1}H-NMR analysis of the synthetic reference compounds. The results obtained identify the various adducts as the 2-glutathionyl-, 2-cysteinylglycyl-, 2-cysteinyl- and 2-*N*-Acetylcysteinyl-5-fluoro-nitrobenzene.

Cytotoxicity of FGNB

To find a non-toxic concentration of FGNB for the studies on the metabolism of FGNB by $LLCPK_1$ and RPT cells, the cellular toxicity of FGNB (25-400 μM) was measured. A significant, cytotoxic effect could be observed after incubation of the $LLCPK_1$ and RPT cell monolayer for 24h with 400 μM FGNB, but not with 200 μM (Fig. 1). In view of this and allowing for a safety margin, metabolism of FGNB by $LLCPK_1$ and RPT cells was investigated at a concentration of 100 μM.

pH dependence of the ^{19}F-NMR chemical shift of the various conjugates.

Fig. 2 shows the effect of medium pH on the chemical shift values of the four synthetic standards. For FGNB and FNAcNB, chemical shift values remain constant over the pH gradient used, indicating a constant protonation/deprotonation ratio of these conjugates at these pH values.

TABLE 1. *Spectral characteristics of 5-fluoro-2-glutathionyl-, 2-cysteinylglycyl-, 2-cysteinyl- and 2-N-acetylcysteinyl-nitrobenzene determined by means of ^{19}F-NMR and ^{1}H-NMR analysis. ^{19}F-NMR in 0.1 M potassium phosphate, pH 7.6, relative to $CFCl_3$. ^{1}H-NMR ($^{2}H_2O$, relative to sodium 3-trimethylsilyl(2,2,3,3-$^{2}H_4$)propionate). Information between brackets refer (1) to the proton or carbon atoms the signal is ascribed to, and (2) to the splitting pattern of the respective NMR resonances: d=doublet, dd=double doublet, t=triplet, m=multiplet; the respective J-coupling constants are also presented.*

5-fluoro-2-(glutathionyl)-nitrobenzene: ^{19}F-NMR (ppm): **-118.5**; ^{1}H-NMR (ppm): 8.02 (H6)(dd, $^{3}J_{H-F}$=8.6 Hz, $^{4}J_{H-H}$= 2.8 Hz), 7.8 (H3)(dd, $^{4}J_{H-F}$= 5.0 Hz, $^{3}J_{H-H}$= 9.1 Hz), 7.58 (H4)(m, $^{3}J_{H-F}$= 7.6 Hz, $^{3}J_{H-H}$= 9.1 Hz, $^{4}J_{H-H}$= 2.8 Hz), 4.65 (CH)(dd, $^{3}J_{H-H}$= 9.3 Hz, $^{3}J_{H-H}$= 4.6 Hz), 3.72 (1H of CH_2)(dd, $^{2}J_{H-H}$= 14.5 Hz, $^{3}J_{H-H}$= 4.6 Hz), 3.37 (1H of CH_2)(dd, $^{2}J_{H-H}$= 14.5 Hz, $^{3}J_{H-H}$= 9.3 Hz), 3.72 (1H of CH_2)(br.s), 3.74 (1H of CH_2)(br.s), 3.61 (CH)(tr, $^{3}J_{H-H}$= 6.4 HZ), 2.04 (CH_2)(tr, $^{3}J_{H-H}$= 7.8 Hz).

5-fluoro-2-(cysteinylglycyl)-nitrobenzene: ^{19}F-NMR (ppm): **-118.5**; ^{1}H-NMR (ppm) 8.04 (H6)(dd, $^{3}J_{H-F}$= 8.6 Hz, $^{4}J_{H-H}$= 2.8 Hz), 7.77 (H3)(dd, $^{4}J_{H-F}$= 5.1 Hz, $^{3}J_{H-H}$= 8.9 Hz), 7.57 (H4)(m, $^{3}J_{H-F}$= 8.8 Hz, $^{3}J_{H-H}$= 8.9 Hz, $^{4}J_{H-H}$= 2.8 Hz), 3.35 (1H of CH_2)(dd, $^{2}J_{H-H}$= 14.7 Hz, $^{3}J_{H-H}$= 5.2 Hz), 3.43 (1H of CH_2)(dd, $^{2}J_{H-H}$= 14.7 Hz, $^{3}J_{H-H}$= 6.1 Hz).

5-fluoro-2-(cysteinyl)-nitrobenzene: ^{19}F-NMR (ppm): **-118.6**; ^{1}H-NMR (ppm): 8.07 (H6)(dd, $^{3}J_{H-F}$= 8.7 Hz, $^{4}J_{H-H}$= 2.9 Hz), 7.74 (H3) (dd, $^{4}J_{H-F}$= 5.1 Hz, $^{3}J_{H-H}$= 9.1 Hz), 7.57 (H4)(m, $^{3}J_{H-F}$= 7.7 Hz, $^{3}J_{H-H}$= 9.1 Hz, $^{4}J_{H-H}$= 2.9 Hz), 3.61 (CH)(dd, $^{3}J_{H-H}$= 7.5 Hz, $^{3}J_{H-H}$= 4.9 Hz), 3.49 (1H of CH_2)(dd, $^{2}J_{H-H}$= 13.3 Hz, $^{3}J_{H-H}$= 4.9 Hz), 3.28 (1H of CH_2)(dd, $^{2}J_{H-H}$= 13.3 Hz, $^{3}J_{H-H}$= 7.5 Hz).

5-fluoro-2-(N-acetylcysteinyl)-nitrobenzene: ^{19}F-NMR (ppm): **-119.1**; ^{1}H-NMR (ppm): 8.01 (H6)(dd, $^{3}J_{H-F}$= 8.6 Hz, $^{4}J_{H-H}$= 2.9 Hz), 7.78 (H3) (dd,$^{4}J_{H-F}$= 5.2 Hz, $^{3}J_{H-H}$=9.2 Hz), 7.57 (H4)(m, $^{3}J_{H-F}$= 7.7 Hz, $^{3}J_{H-H}$= 9.2 Hz, $^{4}J_{H-H}$= 2.9 Hz), 4.44 (CH)(dd, $^{3}J_{H-H}$= 9.0 Hz, $^{3}J_{H-H}$= 4.1 Hz), 3.69 (1H of CH_2)(dd, $^{2}J_{H-H}$= 14.1 Hz, $^{3}J_{H-H}$= 4.1 Hz), 3.32 (1H of CH_2)(dd, $^{2}J_{H-H}$= 14.1 Hz, $^{3}J_{H-H}$= 9.0 Hz), 1.95 (CH_3)(s).

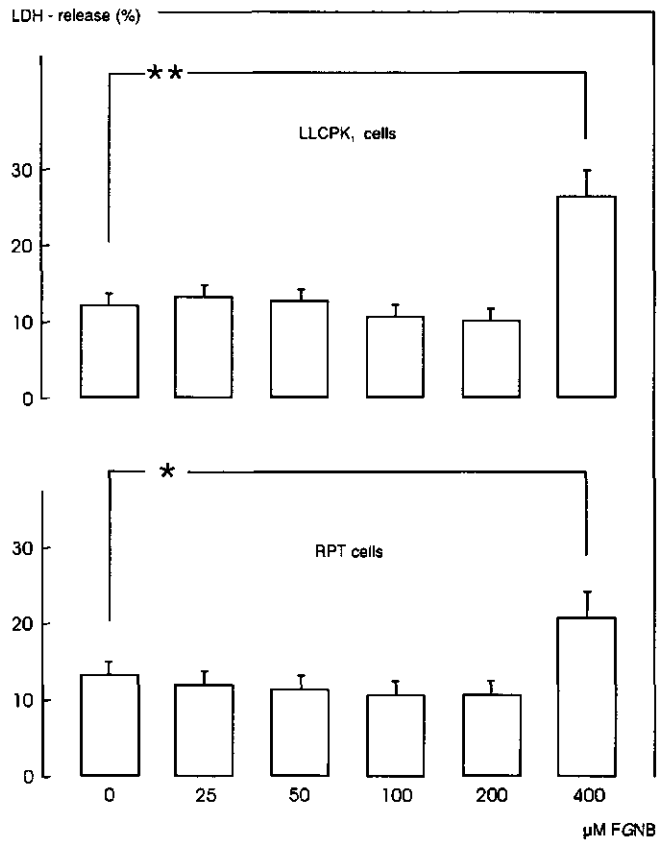

Figure 1. Cytotoxicity of FGNB to LLCPK, and RPT cells. Bars represent the mean (± SEM) of four experiments. Asterisks indicate a statistical difference compared to controls ($p \leq 0.05$ and ** $p \leq 0.02$; Students' t-test).*

The ^{19}F-NMR resonances of both F*CysGly*NB and (at pH 7.5 - 8.5) of F*Cys*NB become more negative with increasing pH, indicating a change in the protonation/deprotonation equilibrium. The results presented in Fig. 2 demonstrate that at pH 7.4 the ^{19}F-NMR chemical shift values of the four thioether conjugates are similar, hampering specific ^{19}F-NMR detection of these conjugates at this pH. Specific detection of all standards was possible at a pH of 8.0 (Fig. 2). Thus, samples were adjusted to this pH-value prior to ^{19}F-NMR analysis. Fig. 3 compares the ^{19}F-NMR spectrum of the synthetic standards at pH 8.0 (Fig. 3a) to the ^{19}F-NMR spectrum of the

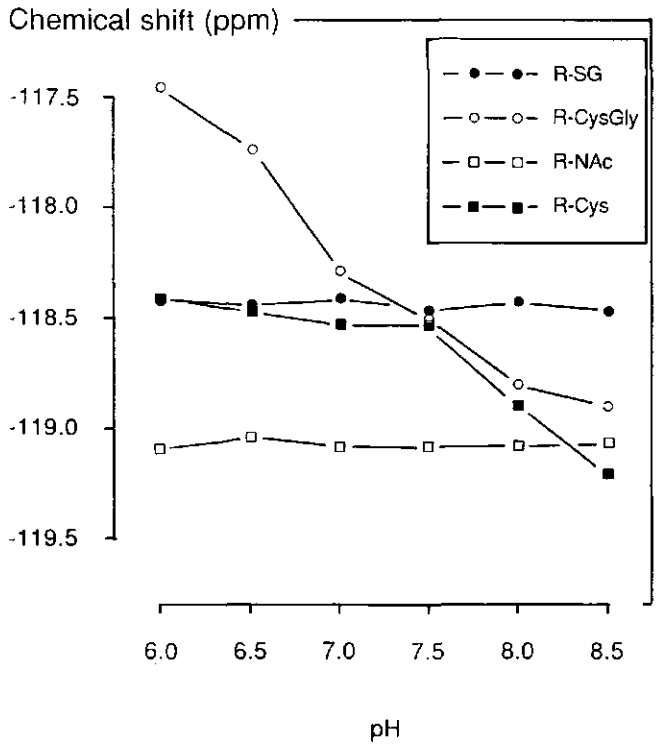

Figure 2. *^{19}F-NMR chemical shift values of FGNB, FCysGlyNB, FCysNB and FNAcNB at different medium-pH. [a](The chemical shifts are relative to $CFCl_3$). [b](R represents 5-fluoro-nitrobenzene).*

medium of LLCPK$_1$ cells exposed to 100 μM F*G*NB for 6h. This figure clearly illustrates that the thioether conjugates formed during incubation of cells with F*G*NB can be selectively detected.

Metabolism of FGNB by LLCPK$_1$ cells

Incubation of a monolayer of LLCPK$_1$ cells with FGNB for 24h resulted in the gradual disappearance of the parent compound (Fig. 4). Concomitantly, two products were formed. From comparison with synthetic standards, one of the products turned out to be F*CysGly*NB. After 12h of incubation, this product reached a maximum concentration of about 20 μM. At 24h, the F*CysGly*NB concentration had declined. The other, (major) end-product was not the mercapturic

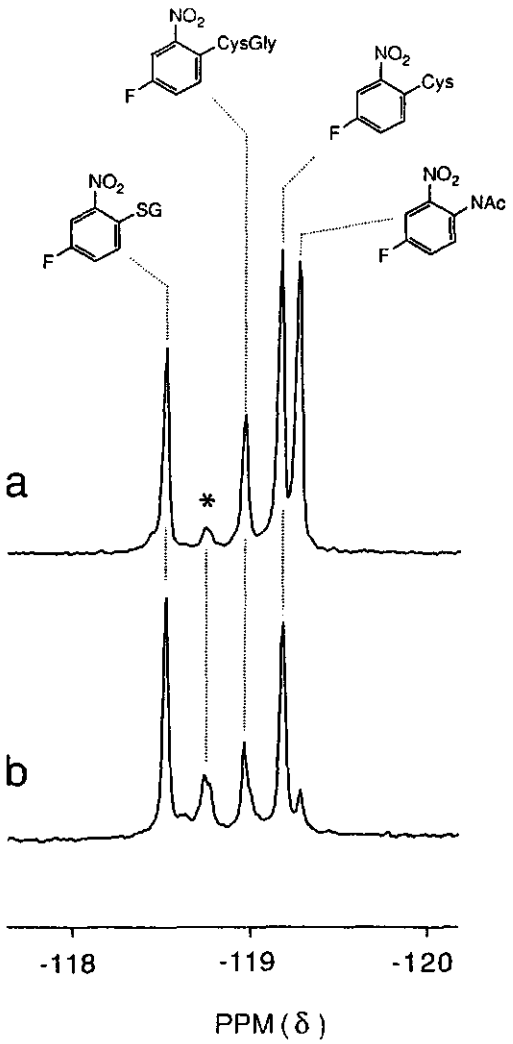

Figure 3. *^{19}F-NMR spectrum of FGNB, FCysGlyNB, FCysNB and FNAcNB at pH 8.0. Synthetic standards (a) and incubation medium (b) of LLCPK$_1$ cells with 100 μM FGNB at 6h. The resonance marked with an asterisk was already present at the start of the incubations and represents an impurity in the preparation.* [a] *(The chemical shifts are relative to $CFCl_3$).*

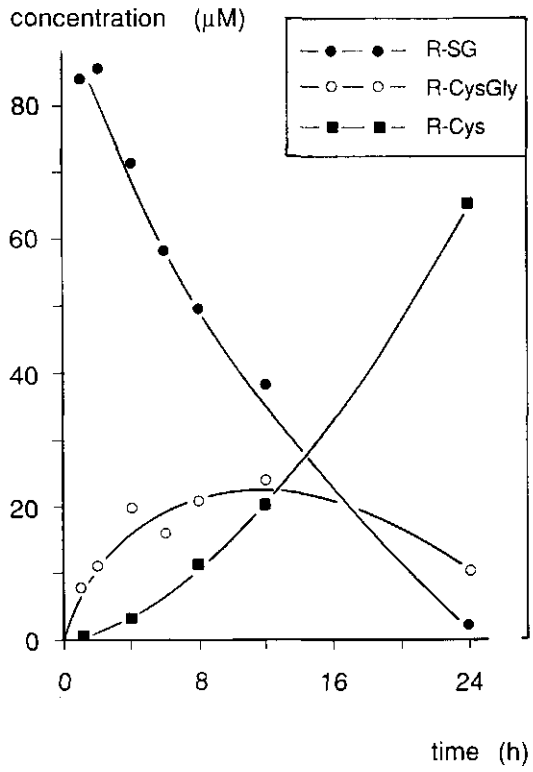

***Figure 4.** Changes in the medium concentration of main thioether conjugates formed during incubation of a LLCPK$_1$ monolayer with FGNB as determined by ^{19}F-NMR. [a](R represents 5-fluoro-nitrobenzene). The results presented are from one representative experiment out of 2.*

acid (F*N*AcNB) but the cysteine-conjugate, F*Cys*NB. This conclusion was drawn from a combination of two observations. Firstly, an extra resonance signal was detected in the spectrum of the 24h medium after spiking the medium with F*N*AcNB standard (Fig. 5 a+b). Secondly, re-neutralisation of the 24h medium, originally set to pH 8.5 for the NMR measurement, resulted in a downfield shift of the ^{19}F-NMR resonance signal of the final metabolite as shown in Fig. 5c. On the basis of the results of Fig. 2, this phenomenon can be expected for the F*Cys*NB but not for the F*N*AcNB.

In addition, incubation of cell lysates of LLCPK$_1$ cells for 24h with 100 μM F*Cys*NB or F*N*AcNB resulted in the recovery of the parent compound (results not shown), suggesting that

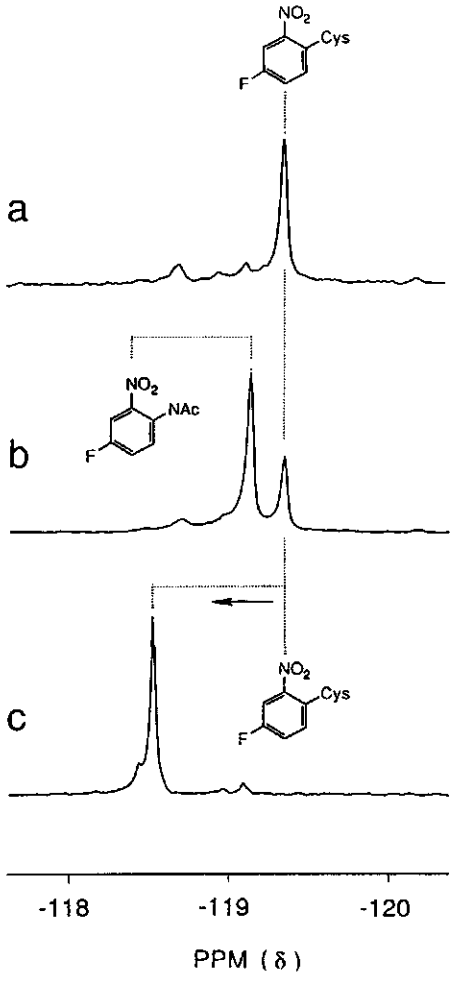

Figure 5. *^{19}F-NMR spectrum of incubation medium of LLCPK$_1$ cells with 100 μM FGNB at 24h (a). ^{19}F-NMR spectrum of 24h medium after addition of exogenous FNAcNB (b). (Note that due to adjustment of the media-pH to 8.5 instead of 8.0, the FNAcNB resonance signal is situated at the lefthand side of the FCysNB resonance signal). Effect of re-neutralisation of 24h medium on FCysNB resonance signal (c).* *[a] (The chemical shifts are relative to $CFCl_3$).*

the cells lack the *N*-acetyltransferase and *N*-deacetylation activity needed for conversion of F*Cys*NB and F*NAc*NB, respectively.

Finally, the results presented in Fig. 6 show that inhibition of β-lyase by AOAA did not influence the biotransformation pattern of FGNB by $LLCPK_1$ cells. Inhibition of γglutamyltranspeptidase (γGT) by acivicin prevented processing of FGNB into FCysNB by the $LLCPK_1$ cells.

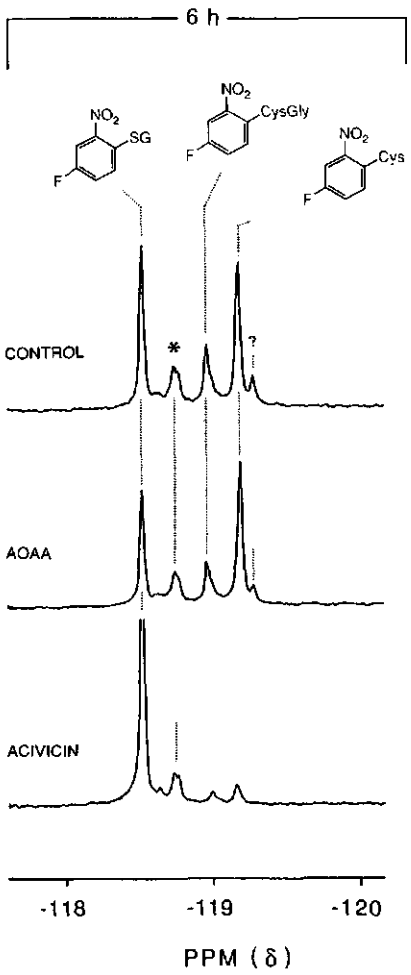

Figure 6. *Effect of AOAA or acivicin on the metabolism of FGNB by $LLCPK_1$ cells at 6h of the incubation. The resonance marked with an asterisk was present at the onset of the incubations.* [a] *(The chemical shifts are relative to $CFCl_3$).*

Metabolism of FGNB by RPT cells

To investigate whether rat RPT cells are also incapable of producing mercapturic acids, we incubated a confluent monolayer of these cells with 100 μM FGNB for 24h. In this incubation too, the cysteine conjugate was formed as the major final metabolite (results not shown).

However, formation of an *N*-acetylcysteine conjugate could be observed when freshly isolated RPT cells were incubated for 24h with FGNB (Fig. 7). Verification of this conclusion was achieved firstly, by spiking the medium with F*Cys*NB, which resulted in a resonance signal at a different ppm value than the resonance signal of the major end product. Secondly, acidification of the F*N*AcNB containing medium to pH 6.2 did not result in a shift of the resonance signal of F*N*AcNB, which is what can be expected for the F*N*AcNB signal on the basis of the results presented in Fig. 2.

Fig. 8 shows that the *N*-acetylation capacity of RPT cells declines dramatically during culture of these cells to confluent monolayers. Only freshly isolated RPT cells and, to a very limited extent, semi-confluent RPT cell monolayers *N*-acetylate cysteine conjugates.

Transport of FCysNB by RPT cells

Since *N*-acetylation of cysteine conjugates occurs intracellularly by microsomal *N*-acetyltransferases (Duffel and Jacoby, 1982), cysteine conjugates have to be taken up by the cell. To exclude that the lack of *N*-acetylation capacity by confluent RPT cell monolayers is the result of a non-functional luminal cysteine-conjugate transporter (Rabito and Karish, 1983), we measured transepithelial transport of F*Cys*NB across confluent RPT cell monolayers on porous supports. No apical to basolateral transport of F*Cys*NB could be demonstrated (results not shown).

DISCUSSION

To date, information on the contribution of kidneys in the metabolism and excretion of halogenated nitro-aromatic compounds is only sparce. Therefore, the present study was undertaken to study proximal tubular metabolism of the glutathionyl conjugate of 2,5-difluoronitrobenzene (FGNB) by means of ^{19}F-NMR.

Due to the fact that differences in molecular structure of the various 2,5-DFNB derived metabolites concern only the aliphatic part of the molecule, which is at several bonds distances from the F atom, the ^{19}F-NMR resonances of the four conjugates are minimally influenced by a chemical modification of this side chain. As a result, the ^{19}F-NMR resonances lie within a

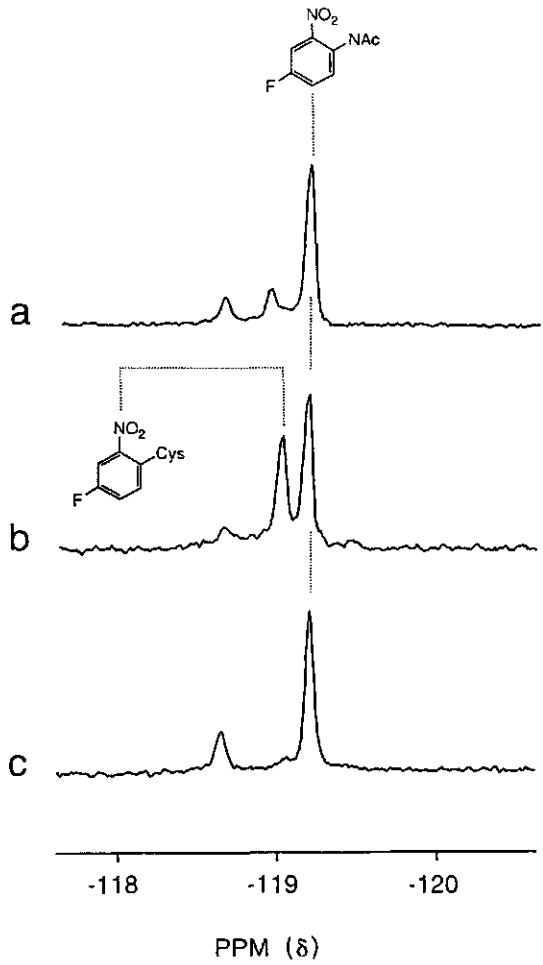

Figure 7. *^{19}F-NMR spectrum of incubation medium of freshly isolated proximal tubular cells with 100 μM FGNB at 24h (a). ^{19}F-NMR spectrum of 24h medium after addition of exogenous FCysNB (b). Effect of acidification of 24h medium to pH 6.2 on FNAcNB resonance signal (c). [a](The shifts are relative to $CFCl_3$).*

narrow ppm-range. However, the chemical shift of the four synthetic standards in incubation medium could be influenced by minor modifications in pH. Thus, selective detection of fluorinated thioether conjugates could be obtained at an increased pH value e.g. (8.0).

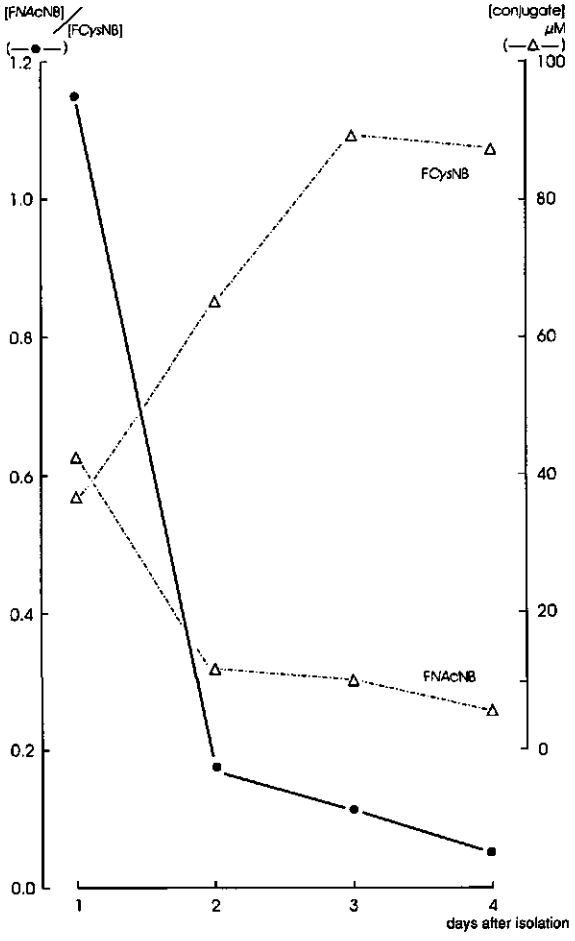

Figure 8. *N-acetylation capacity of cultured RPT cells at different times after isolation. RPT cells were exposed for 24h to 100 μM FGNB in WE medium without serum immediately after seeding and after 1, 2 and 3 days of culture. At the end of the incubation, media were adjusted to pH 8.0 and stored at -20°C until ^{19}F-NMR analysis. The results presented are from one representative experiment out of three.*

Incubation of LLCPK$_1$ monolayers with FGNB for 24h showed that the concentration of FGNB gradually declined. Concomitantly, a cysteinylglycine conjugate is formed together with a

cysteine conjugate (Fig. 4). Inhibition of β-lyase by AOAA did not influence the biotransformation pattern of F*G*NB by $LLCPK_1$, suggesting that this enzyme is not involved in the biotransformation of F*G*NB (Fig. 6). Since acivicin prevents γGT-mediated cleavage of the glutathione conjugate into a *CysGly* conjugate, as expected, no F*CysGly*NB was formed in this incubation. These results are in agreement with the biotransformation rates of GSH and GSH conjugates studied with rat renal brush border membrane vesicles (Okajima *et al.*, 1981) and isolated kidney cells (Jones *et al.*, 1979). However, in our study F*CysGly*NB is formed faster than it is hydrolysed into F*Cys*NB. As a result, an initial build up of the concentration of F*CysGly*NB can be observed. It is generally accepted that cysteine conjugates such as F*Cys*NB can be *N*-acetylated both in the liver and kidney to the presumed endproduct: the mercapturic acid (F*N*AcNB). Surprisingly, in our experiments with $LLCPK_1$ cells this F*N*AcNB conjugate could not be observed as the final product suggesting a low *N*-acetylating capacity of these cells. This conclusion is supported by the fact that incubation of cell lysates of $LLCPK_1$ cells with either F*Cys*NB or F*N*AcNB only resulted in the recovery of the parent compound (results not shown). The possibility that the *N*-acetyltransferase and *N*-deacetylase enzymes have lost their activity during the preparation of the cell lysate cannot be completely ruled out, since *N*-acetyltransferases have been reported to be labile in subcellular systems (Duffel and Jacoby, 1982). Additional studies with RPT cells further corroborated the explanation that the absence of *N*-acetylation of F*Cys*NB can be ascribed to the loss of *N*-acetyltransferase activity. Incubation of freshly isolated RPT cells with F*G*NB resulted in the formation of F*N*AcNB as major metabolite demonstrating the capacity of these cells to *N*-acetylate F*Cys*NB to give the mercapturic acid. However, upon cultivation of the cells this capacity was lost and F*Cys*NB was again found as the major, final product of F*G*NB metabolism.

Thus, a low *N*-acetylating capacity is not restricted to $LLCPK_1$ cells, but is also observed for cultured RPT cells. This observation for the RPT cells is in accordance with the results of Boogaard *et al.* (1989) who reported little formation of mercapturic acids after incubation of RPT cells with cysteine conjugates of dichlorodifluoroethylenes (DCDFE). Whereas when *in vivo* administered to rats, they were recovered in urine for 60% as mercapturates (Commandeur *et al.*, 1987). Possible inactivation of *N*-acetyltransferases during isolation of RPT cells or a depletion of acetyl-coenzyme A, an important cofactor in the activity of *N*-acetyltransferases, were thought to be responsible for the observed discrepancy in results (Boogaard *et al.*, 1989). Since 19% of 2,5-difluoronitrobenzene is metabolised *in vivo* in rats into a *N*-acetylcysteine conjugate and no cysteine conjugate is excreted (Rietjens *et al.*, 1995), and because freshly isolated RPT cells are able to form the *N*-acetylcysteine conjugate, the above mentioned possibilities may also account for the discrepancy in the *in vitro* and *in vivo* metabolism pattern of F*G*NB.

Alternatively, the fact that apical to basolateral transport of F*Cys*NB by RPT cell monolayers could not be demonstrated suggests that a deficiency in cellular uptake of F*Cys*NB by cultured $LLCPK_1$ and RPT cells may provide an alternative and/or additional explanation for the lack of mercapturic acid formation.

Altogether, the results of the present study illustrate a method to study the metabolism of a model glutathione conjugate through the mercapturic acid pathway by means of ^{19}F-NMR. This method was sensitive enough to detect metabolites of which the chemical modification was more than 8 σ-bonds away from the fluorine atom. The results obtained also demonstrate that $LLCPK_1$ cells as well as cultured RPT cells lack the capacity to perform the conversion of the cysteine adduct into the mercapturic acid. This capacity was still present in freshly isolated RPT cells incubated in suspension. Additional data of the present study demonstrate that this deficiency can at best be ascribed to a loss of either the *N*-acetyltransferase enzyme and/or the transport systems which are needed for the uptake of the F*Cys*NB conjugate into the cell where the *N*-acetyltransferases are located, all within 24h of cultivation.

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CHAPTER 6

Comparison of the viability and metabolic capacity of freshly isolated and cryopreserved rat kidney precision-cut cortical slices: para-aminohippuric acid uptake and mercapturic acid formation from 5-fluoro-2-(glutathionyl)-nitrobenzene

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Xenobiotica (submitted)

SUMMARY

The ability of freshly isolated and cryopreserved rat kidney precision-cut cortical slices to perform basic reactions in xenobiotic metabolism was compared using para-aminohippuric (PAH) acid uptake and mercapturic acid formation from 5-fluoro-2-(glutathionyl)-nitrobenzene (F*G*NB) as the model reactions.

Characterization of viability by measuring lactate dehydrogenase (LDH)-leakage) indicated that viability was substantially lower for cryopreserved rat renal cortical slices than for freshly isolated renal slices.

PAH acid accumulation, indicative for organic anion transport, shows a 65% reduction in cryopreserved renal cortical slices compared to freshly isolated slices after 4h of culture. However, the relative reduction in PAH uptake observed for both freshly isolated and cryopreserved renal cortical slices from 4h to 24h of incubation, was similar. This implies that the cryopreserved renal cortical slices can still provide a valuable tool to assess the effect(s) of xenobiotics on organic anion transport.

Cryopreservation does not affect γglutamyltranspeptidase (γGT) and dipeptidase-mediated formation of 5-fluoro-2-(cysteinyl)-nitrobenzene (F*Cys*NB).

The capacity for *N*-acetylation of F*G*NB by both freshly isolated and cryopreserved rat renal cortical slices is comparable for up to 4h. At 6h and 24h, the *N*-acetylation capacity was substantially lower in cryopreserved renal slices. This indicates that cryopreservation might affect the enzymes involved.

To study possible influences of species variation, part of the experiments were repeated using cryopreserved beagle dog renal cortical slices. For these slices, similar ratios between 5-fluoro-2-

(*N*-acetylcysteinyl)-nitrobenzene (F*N*AcNB) and F*Cys*NB were observed as for the rat cryopreserved slices, suggesting that beagle dog renal cortical slices can also be used to study *N*-acetylation of xenobiotics but may have a similar problem with stabilisation of the enzymes in time.

In conclusion, the data of the present study show that cryopreserved precision-cut renal slices can provide a valuable tool for assessing *in vitro* toxicity, although one has to take into account that two important processes in the bioactivation and detoxicifation of xenobiotics i.e. organic anion transport and *N*-acetylation are affected by the cryopreservation procedure.

INTRODUCTION

The use of precision-cut renal cortical slices for testing *in vitro* metabolism and toxicity of xenobiotics provides a system which may serve as a valuable tool for predicting *in vivo* metabolic and toxicological responses (Smith, 1988; Wolfgang *et al.*, 1989; Trevisan *et al.*, 1992). Compared with isolated renal cortical cells, renal slices retain the multicellular composition and histological orientation of the tissue as well as intact membrane receptors and transporters (Berndt, 1976).

In most cases, tissue slices were incubated in a dynamic organ culture system under an atmosphere of 95 - 100% O_2, as developed by Smith *et al.* (1982). In other studies, the amount of atmospheric oxygen has been reduced to 40% (Wright and Paine, 1992; Leeman *et al.*, 1995) in order to minimize oxidative damage, especially during prolonged culture periods.

To establish both a reduction in the use of animals and a prolongation of the useful lifetime of both animal and human tissue for experiments, tissues can be cryopreserved. For that purpose, several methods were developed (Fahy *et al.*, 1984; Powis *et al.*, 1987; Fisher *et al.*, 1993). For cryopreserved tissue to act as a valid system for toxicity and metabolism studies, it must, after thawing, maintain all biotransformation reactions at levels comparable to those in freshly prepared tissue. In this respect, research has initially focussed on the usefulness of cryopreserved liver tissue (Powis *et al.*, 1987; Coundouris *et al.*, 1990; Wishnies *et al.*, 1991). To date, the usefulness of cryopreserved renal tissue for studying toxicity and metabolism of xenobiotics is also under investigation. Fisher and coworkers (1993) assessed the viability of cryopreserved human kidney slices by measuring potassium content, protein synthesis and organic anion and cation uptake using the model compounds PAH and tetraethylammonium (TEA), respectively. In addition, Kruidering *et al.* (1994) studied cellular viability of cryopreserved porcine proximal tubular cells using flow cytometric analysis and by measuring the mitochondrial membrane

potential of these cells.

In this study, we compared the validity of freshly isolated and cryopreserved rat renal cortical slices as model systems for studies on the biotransformation of xenobiotics by studying a basic metabolic process of importance for renal xenobiotic metabolism, i.e. mercapturic acid formation. In addition, since renal metabolism of xenobiotics is often associated with cellular uptake of these compounds as organic anions (Pritchard and Miller, 1993), uptake of PAH is also included in this study. Since kidneys metabolise glutathione conjugates into mercapturic acids (Monks and Lau, 1987), the activity of the mercapturic acid pathway is an important parameter for assessing the usefulness of cryopreserved renal cortical slices for toxicity and metabolism studies. The model reaction used to investigate this biotransformation capacity of freshly isolated and cryopreserved rat renal cortical slices was metabolism of a glutathione conjugated nitrobenzene compound: 5-fluoro-2-(glutathionyl)-nitrobenzene (FGNB), known to undergo metabolism via the mercapturic acid pathway (Haenen *et al.*, 1995). For comparison with another mammalian system, the capacity of cryopreserved beagle dog renal cortical slices to form mercapturic acids was also studied.

MATERIALS AND METHODS

Chemicals

2,5-Difluoronitrobenzene, glutathione (GSH) and *N*-acetyl-L-cysteine were obtained from Janssen (Beerse, Belgium). Cysteinylglycine, L-cysteine, acivicin and probenecid were bought from Sigma (St. Louis, MO, USA). Sodium pyruvate and NADH were from Boehringer (Mannheim, Germany). p-[*glycyl*-1-^{14}C]-aminohippuric acid (1.63 GBq/mmol, radiochemical purity >97%) was purchased from New England Nuclear (Dreiech, Germany). Hanks Balanced Salt Solution (HBSS) without phenol red was obtained from Flow (Irvine, Scotland). Foetal bovine serum (FBS) was obtained from Integro B.V. (Zaandam, The Netherlands). DMEM with 25 mM Hepes, HAM's F12 and Williams medium E (WE) supplemented with glutamax I, phosphate-buffered saline (PBS) and gentamicin (50 mg/ml) were obtained from Gibco (Paisley, Scotland). All other chemicals were of the highest purity commercially available.

Animals

For experiments, female Wistar rats (Harlan CPB, Zeist, The Netherlands) weighing 180-230 g were used. The animals had free access to food and tap water and were kept on a 12h/12h day/night cycle.

Conjugate synthesis

5-Fluoro-2-(glutathionyl)-nitrobenzene (F*G*NB), 5-fluoro-2-(cysteinylglycyl)-nitrobenzene (F*CysGly*NB), 5-fluoro-2-(cysteinyl)-nitrobenzene (F*Cys*NB) and 5-fluoro-2-(*N*-acetylcysteinyl)-nitrobenzene (F*N*AcNB) were synthesized from 2,5 difluoronitrobenzene (2,5-DFNB) and characterised as described previously (Haenen *et al.*, 1995).

Preparation of renal slices

Animals were anaesthesized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Kidneys were dissected and 5 mm diameter cortical biopsies were obtained using a disposable biopsy punch. Kidney slices (200 - 250 μm) were prepared at 4°C using a Krumdieck tissue slicer (Krumdieck *et al.*, 1980) in DMEM/HAM's F12 (1:1 v/v) supplemented with 25 mM HEPES and gentamicin (50 μg/ml). Slices were placed on 200 μm Netwell inserts (2 slices per insert) in 6-well cell culture clusters (Costar, Badhoevedorp, The Netherlands) containing 3.25 ml DMEM/HAM's F12 supplemented with 25 mM D-glucose, 5% FBS and 50 μg/ml gentamicin (culture medium). The tissue culture clusters were placed in a humidified incubator at 37°C, 40% O_2 and 5% CO_2 on a rocker platform (approximately 10 cycles per minute). For about half the cycling time slices were immersed in incubation medium or exposed to air.

Cryopreservation

Cryopreservation of slices was perfomed using a SY-LAB ice Cube 1610 Scientific Biological Freezer (Holland Cryogenic, Int., Hedel, The Netherlands). Slices were kept on ice in cryo vials containing culture medium. While keeping the cells on ice, 10% DMSO was added slowly. The vials were put in the freezing chamber and both the sample and chamber temperature were monitored. The applied cooling rate was -0.5°C/min. To prevent a rise in sample temperature at the crystallisation temperature, the temperature in the chamber was decreased at maximum rate (8°C/min). After reaching a sample temperature of -80°C, the vials were stored in liquid nitrogen. For experiments, cryopreserved slices were thawed in a 37°C waterbath.

PAH uptake experiments

Prior to PAH uptake, freshly prepared and cryopreserved slices were pre-incubated in culture medium for 1h. Subsequently, slices were incubated in culture medium without serum for 3h and 23h, repectively. Netwells were transferred to 6-well cell culture clusters containing 78 μM PAH in HBSS spiked with 0.5 μCi [^{14}C]-PAH/well and incubated for 1h. PAH in slices and medium was determined and expressed as slice (S) to medium (M) ratio i.e. the concentration of the label in the slice (expressed as mmol/g wet weight) compared to the concentration of the label in the medium (expressed as mmol/ml) as described by Berndt (1976). 100 μL of incubation solution was counted in a liquid scintillation counter in triplicate. The slices were washed in HBSS,

blotted dry and weighted. After this, they were transferred in a counting vial and 0.5 ml tissue solubilizer (Soluene-350, Packard) and 15 μl H_2O_2 (30% v/v) was added. After an overnight incubation at room temperature, 100 μl of this solution was counted in triplicate in a liquid scintillation counter after addition of 4 ml Hionic Fluor (Packard).

LDH-leakage

Cytotoxicity was assessed by measuring LDH-leakage in 150 μl of incubation medium according to a method by Mitchell *et al.* (1980). LDH-leakage was calculated as the decrease in [NADH] in mM per min.

Metabolism experiments

Kidney cortical slices were pre-incubated for 1h in culture medium with FBS. After this period, the medium was replaced by culture medium without FBS containing 400 μM FGNB. Freshly prepared and cryopreserved slices were incubated as described for 24h. At indicated time points samples of the medium were drawn, pH set to 8.0 and stored at -20°C until ^{19}F-NMR analysis as described by Haenen *et al.* (1995). To monitor the intracellular content of thioether conjugates, slices were washed in HBSS, blotted dry on filtration paper and weighed. In addition, slices were homogenized in a Potter homogenizer in ice-cold DMEM/HAM's F12. The homogenate was centrifuged for 10 minutes at 10.000*g* in an Eppendorf centrifuge and the supernatant was stored at -20°C until ^{19}F-NMR.

Statistics

When appropriate, data are expressed as means $\pm$ SD of at least three experiments. Statistical comparisons were made between groups using the student's *t*-test (one tailed). Significance was accepted at $p \leq 0.05$.

RESULTS

LDH-leakage

In order to assess the viability of the freshly isolated and cryopreserved rat renal cortical slices and to test a non-cytotoxic concentration to study metabolism of FGNB, we measured LDH-leakage from these slices without and upon exposure to FGNB (Fig. 1). The LDH-leakage in cryopreserved slices was always higher than LDH-leakage in the corresponding incubations with freshly isolated slices. However, this did not affect the possibility to use the cryopreserved slices

to test the cytotoxicity of FGNB. This can be derived from the observation, also presented in Fig. 1, that after 4h and 24h of incubation of freshly isolated and cryopreserved renal slices with or without 400 μM FGNB, LDH-leakage did not differ.

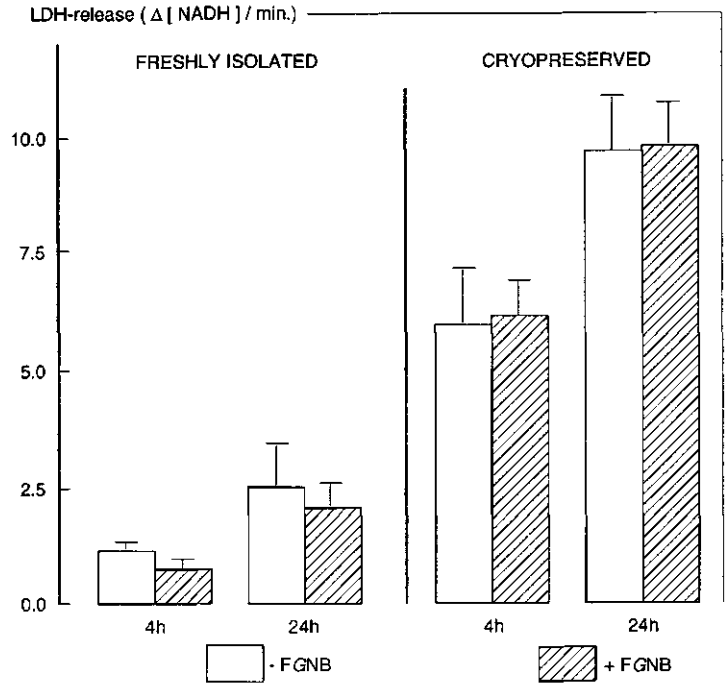

Figure 1. Effect of 400 μM FGNB on LDH-release from freshly isolated and cryopreserved rat renal precision-cut cortical slices after 4h and 24h of incubation. Results (mean ± SEM) are expressed as the decrease in concentration of NADH in mM/min of three independent experiments.

PAH uptake

The viability of freshly isolated and cryopreserved rat renal cortical slices was assessed by measuring PAH uptake by these slices. The accumulation of PAH by freshly isolated and cryopreserved renal slices after 4h and 24h of pre-incubation are compared in Table 1.

Table 1. PAH uptake in freshly isolated and cryopreserved rat renal precision-cut cortical slices. Results (mean ± SD of three experiments in triplicate) are expressed as slice to medium (S/M) ratio and as % of the S/M value at 4h and 24h.

	freshly isolated renal cortical slices		cryopreserved renal cortical slices	
time (h)	(S/M)	(%)	(S/M)	(%)
4	4.5 ± 1.6	100 ± 36	1.6 ± 0.6	100 ± 38
24	3.2 ± 2.2	71 ± 49	1.1 ± 0.8	69 ± 50

Cryopreserved slices accumulate considerably less PAH than freshly isolated slices and the S/M ratio in cryopreserved slices was generally 35% of the value observed for freshly isolated slices. Comparison of the S/M values observed at 4h and 24h of incubation showed that for both type of slices uptake at 24h was reduced to about 70% of the value observed at 4h.

The inhibitor probenecid (500 μM) was used to demonstrate that PAH uptake reflects an active anionic transport system (Lash and Jones, 1985). As expected, pre-incubation of freshly isolated renal slices with probenecid significantly ($p \leq 0.01$) decreased PAH uptake (0.82 ± 0.25; n=3).

Metabolism of FGNB by renal cortical slices

The effect of cryopreservation on renal xenobiotic metabolism was further investigated by measuring the ability of freshly isolated and cryopreserved precision-cut renal slices to metabolise F*G*NB, the glutathione conjugate of 2,5-difluoronitrobenzene. The results are expressed in Table 2 as the medium concentrations of the two final products formed and as the ratio of these products, i.e. F*N*AcNB, the mercapturic acid, and F*Cys*NB, its non-acetylated precursor. During the first 4h of incubation the amount of mercapturic acid formed is comparable in both freshly isolated and cryopreserved slices. However, after 6h and 24h both the concentration of F*N*AcNB and the [F*N*AcNB]/[F*Cys*NB] ratio is approximately 1.5-fold higher in media of freshly isolated slices than in media of cryopreserved renal slices. The results in Table 2 also demonstrate that at prolonged incubation times (6h and 24 h) a larger amount of F*Cys*NB is formed in media of cryopreserved renal slices than in media of freshly isolated renal slices, suggesting that

cryopreservation does not affect the activity of γGT and dipeptidases.

For comparison, an identical experiment was carried out with cryopreserved dog precision-cut renal slices. The [F*N*AcNB]/[F*Cys*NB] ratio increased to maximally 0.28 after 24h of incubation of these slices to 400 μM F*G*NB, indicating a similar time course in mercapturic acid formation as with cryopreserved rat renal slices. Together, these data indicate that the γGT and dipeptidase mediated formation of the cysteine adduct is not affected in cryopreserved rat precision-cut renal slices. However, its *N*-acetylation capacity may be seriously impaired.

Table 2. *Time course in cysteine conjugate and mercapturic acid formation after metabolism of 400 μM FGNB by freshly isolated and cryopreserved rat renal cortical slices. Results are expressed as concentrations (in μM) of FCysNB and FNAcNB formed and as the ratio [FNAcNB]/[FCysNB] of one experiment typical of two.*

	freshly isolated renal cortical slices			cryopreserved renal cortical slices		
time (h)	[F*Cys*NB]	[F*N*AcNB]	ratio	[F*Cys*NB]	[F*N*AcNB]	ratio
2	135	29	0.22	141	28	0.20
4	181	37	0.20	137	29	0.21
6	173	43	0.25	207	31	0.15
24	161	70	0.43	194	48	0.25

DISCUSSION

To date, knowledge on the usefulness of cryopreserved renal tissue as a tool for predicting *in vivo* metabolic and toxicological responses is limited. In this study, we provide information on this matter by comparing cytotoxicity, organic anion uptake and the capacity of mercapturic acid synthesis by cryopreserved and freshly isolated renal slices. Although cryopreserved renal slices

and cells were previously used to detect toxicity (Fisher *et al.*, 1993; Kruidering *et al.*, 1994), to date no study focussed on the effect of cryopreservation on mercapturic acid formation, a process of importance for biotransformation and toxicity of compounds.

The initial release of LDH from freshly isolated renal slices is mainly the result of renal cell damage at the cutting edge of the slices. The increase in LDH-leakage after 24h of incubation, however, is the result of a decrease in cellular viability of the entire slice during culture. Cryopreserved renal slices show an almost 6-fold increase in LDH-leakage which is, obviously, the result of cellular damage due to the freeze/thaw procedure.

FGNB (400 μM) was not cytotoxic to both freshly isolated and cryopreserved renal cortical slices. In contrast to this, LDH-leakage was observed after incubation of rat renal proximal tubular (RPT) cell monolayers to 400 μM of FGNB (Haenen *et al.*, 1995). The absence of a toxic effect of FGNB in precision-cut renal slices might be related to the used concentration of FGNB that does not reach all RPT cells in renal cortical slices. Alternatively, the disruption of RPT cells from their histological orientation as well as the concomitant deleterious effects of ischaemia, enzymatic digestion and probably dedifferentiation, makes (cultured) RPT cells more vulnerable than RPT cells in cortical slices.

In contrast to LDH-leakage, PAH accumulation is generally considered to be a more sensitive and specific functional parameter for proximal tubular damage. The slice to medium (S/M) ratio observed in our experiments using female Wistar rats was similar to the value observed for rabbit renal cortical slices (Wolfgang *et al.*, 1989) and human kidney slices (Fisher *et al.*, 1993). However, the S/M ratio measured in our experiments was relatively low when compared with results by others who measured a 3-6 times higher S/M ratio by rat renal cortical slices (Smith *et al.*, 1982; Matsushima and Gemba, 1982). Incubation of slices with culture medium in the presence of serum before PAH uptake did not augment the slice to medium ratio in our experiments (results not shown). This might suggest that proteolytic enzymes released from the damaged cells do not affect PAH uptake mechanisms and hence are not responsible for the relatively lower slice to medium ratio. Since it was shown that acetate stimulates PAH uptake *in vitro* and enhances respiration of renal cells (Cross and Taggart, 1950), the presence of acetate in the medium used by Smith *et al.* (1982) and Matsushima and Gemba (1982) might be an important factor in the higher PAH uptake in their experiments.

As a result of extended cellular damage, cryopreserved rat renal cortical slices show a 65% reduction in PAH accumulation compared to freshly isolated slices. In addition, PAH uptake in cryopreserved human renal slices was only 20% lower than in freshly isolated slices (Fisher *et al.*, 1993). From this it might be concluded that human renal slices are less sensitive to cryopreservation induced cell damage than rat renal slices. Nevertheless, the observation that both freshly isolated and cryopreserved rat renal slices show a comparable relative decline in

PAH uptake going from 4h to 24h of incubation, suggests that cryopreserved slices are, in spite of lower overall S/M accumulation ratio's, a useful tool for measuring the effects of xenobiotics on PAH accumulation.

Both cryopreserved and freshly isolated rat renal slices metabolise F*G*NB into its respective cysteine conjugate (F*Cys*NB) and *N*-acetylcysteine conjugate (F*N*AcNB). As judged from the amount of F*Cys*NB formed in both incubations, it seems that cryopreservation does not impair the activity of the enzymes involved: γGT and dipeptidases. However, cryopreservation does seem to have an inhibitory effect on the *N*-acetylation capacity of rat renal slices. After 6h of incubation, a larger amount of F*Cys*NB and a lower amount of F*N*AcNB was observed in media of cryopreserved rat renal slices compared to media of freshly isolated cells. A similar effect was observed using beagle dog renal slices. Moreover, we have shown that the *N*-acetylation capacity of rat renal proximal tubular cells declines rapidly during culture (Haenen *et al.*, 1995). Since *N*-acetyltransferase is a cytosolic enzyme (Duffel and Jacoby, 1982), it might have lost some activity during the freeze/thaw session. In agreement with this, Coundouris *et al.* (1990) observed a decline in activity of the cytosolic enzymes glutathione *S*-transferase and glutathione reductase in cryopreserved rat hepatocytes whereas the activity of microsomal enzymes like cytochromes P-450 were not affected by cryopreservation.

In summary, cryopreservation affects the absolute amount of organic anion accumulation by renal cortical slices but does not seem to affect the possibility to judge effects of culture conditions and/or toxic compounds on organic anion accumulation. In addition, as judged from the decreased *N*-acetylation capacity in cryopreserved renal slices, cryopreservation affects cytosolic *N*-acetyltransferase activity leading to decreased formation of mercapturic acids and increased accumulation of the corresponding cysteine conjugate. Because cysteine conjugates can be bioactivated through the activity of β-lyase (Anders *et al.*, 1988), this may influence the toxicity of compounds known to be bioactivated via the β-lyase pathway.

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CHAPTER 7

Summary and concluding remarks

Summary

Renal proximal tubular (RPT) cells can be exposed apically to glomerulary filtrated and basolaterally to non-filtrated nephrotoxic compounds. To excrete these compounds via the urine, RPT cells are equipped with transport systems able to transport nephrotoxicants from the basolateral to the apical side of the tubule. Since the majority of *in vitro* models used so far to study nephrotoxicity concern suspensions of RPT cells or RPT cells cultured on solid supports, in which transport systems are only partially or no longer active, less attention has been paid to the role of these transport systems in renal toxicity. An adequate assessment of RPT toxicity of nephrotoxicants should include the role of functional transport systems. Therefore, the aim of the present thesis was to develop such an *in vitro* model. We cultured rat RPT cells to confluency on porous supports of tissue culture inserts. Monolayers of RPT cells remained confluent for 24h. After that time, they became leaky and cell viability gradually declined (Chapter 2).

The glutathione (GSH) conjugates of (hydro)quinones, nitrobenzene and ethacrynic acid were used as model compounds. *In vivo*, these compounds are targetted to RPT cells since these cells are equipped with both enzyme and transport systems that use these potentially toxic glutathione conjugates as substrate.

The glutathione conjugate of the quinoid compound menadione (MGNQ) was only toxic after basolateral challenge of confluent monolayers of RPT cells (Chapter 2). By preventing basolateral uptake of this compound via organic anion systems, an increase in cytotoxicity was observed. Similar effects were observed when basolateral uptake and subsequent deacetylation of M(NAC)NQ, the *N*-acetylcysteine conjugate of menadione, were inhibited. It is generally agreed upon that γglutamyltranspeptidase (γGT) and dipeptidase mediated metabolism of GSH conjugates into cysteine conjugates is a prerequisite for cellular uptake of the latter conjugates. MGNQ induced apical cytotoxicity only after inhibition of apical γGT. These observations suggest that cellular uptake of thioether conjugates is associated with detoxification. The postulated reaction mechanism resulting in detoxification of the GSH and *N*-acetylcysteine conjugate of menadione is drawn in Fig. 4 (Chapter 2).

Detoxification of quinone cystein(glycinyl) conjugates can occur via an intramolecular cyclization reaction which eliminates the reactive quinone function (Monks *et al.*, 1990). The identification and characterisation of the cyclization product of MGNQ provided evidence that this reaction is responsible for the detoxification of MGNQ by RPT cells in this *in vitro* system

(Chapter 4).

In agreement with results obtained with MGNQ, both mono-substituted glutathione conjugates of 2-*tert*-butyl hydroquinone (5SG-TBHQ and 6SG-TBHQ) were only toxic after basolateral exposure of RPT cell monolayers (Chapter 3). Apparently, at this side of the monolayer γGT-mediated metabolism of GSH conjugates is probably not sufficient for complete detoxification of these conjugates in this *in vitro* system (Chapters 2 and 3). In addition, the di-substituted GSH conjugate of TBHQ (3,6SG-TBHQ) was not cytotoxic in our system. In line with this, 3,6SG-TBHQ has a lower ability to generate reactive oxygen species compared with both mono-substituted conjugates (Van Ommen *et al.*, 1992), suggesting that the (hydro)quinone induced proximal tubular toxicity mainly results from redox cycling (Chapter 3). Since superoxide dismutase and catalase, which cannot be transported into the cell, completely abolished SG-TBHQ induced cytotoxicity, quinol-thioether induced redox cycling is likely to take place extracellularly (Chapters 2 and 3).

When RPT cells were cultured on solid supports, apical exposure to 5SG-TBHQ induced a cytotoxic effect whereas 6SG-TBHQ did not. Inhibition of γGT completely abolished 5SG-TBHQ induced cytotoxicity, whereas a cytotoxic effect was observed with 6SG-TBHQ. This result underlines the concept that γGT on the one hand initiates detoxification of GSH-conjugated (hydro)quinones by removing the reactive quinone function (5SG-TBHQ) and on the other hand it gives rise to conjugates that can undergo redox cycling more easily (6SG-TBHQ) (Chapter 3).

No transepithelial transport of MGNQ or metabolites thereof could be observed across RPT cell monolayer. Most likely, transport occurred via paracellular leakage as a result of a reduction in monolayer integrity due to toxicity via extensive redox cycling of the quinone under the culture conditions. Also, no basolateral to apical transport of the GSH conjugate of ethacrynic acid, an α,β unsaturated ketone (EASG), was observed across the RPT cell monolayer. Probably, the transporter responsible for transport of this conjugate lost its function in our model. In contrast, only apical to basolateral transport of EASG was observed. Since acivicin reduced this transport of EASG and inhibited the formation of free, unconjugated ethacrynic acid (EA), not the GSH conjugate is transported but rather the cysteine conjugate and/or free EA (Chapter 4).

In Chapter 5 it was observed by means of ^{19}F-NMR that monolayers of both LLCPK$_1$ cells and primary RPT cells were not able to metabolise the GSH conjugate of 2,5-difluoronitrobenzene (F*G*NB) into its *N*-acetylcysteine derivative (F*N*AcNB). Consequently, the cysteine conjugate of 2,5-difluoronitrobenzene (F*Cys*NB) was the major metabolite formed. However, freshly isolated RPT cells were able to form mercapturic acids but, upon cultivation, quickly lose this capacity. In addition, no apical to basolateral transport of the cysteine conjugate of 2,5-difluoronitrobenzene (F*Cys*NB) was observed across monolayers of primary RPT cells (Chapter 5).

In Chapter 6 we studied the ability of another *in vitro* renal cell system i.e. freshly isolated

renal cortical slices to metabolise xenobiotics by measuring their *N*-acetylation capacity. We observed that these slices metabolised F*Cys*NB into the mercapturate F*N*AcNB. To find out whether cryopreserved rat renal cortical slices could be used as an alternative for freshly isolated tissue in metabolism studies, we compared their biotransformation capacity. For up to 4 h, the *N*-acetylation capacity of both type of slices is comparable. Thereafter, the *N*-acetylation capacity is substantially lower in cryopreserved tissue. Since cryopreserved rat renal cortical slices did not metabolise F*G*NB into a lower amount of F*Cys*NB than freshly isolated tissue, cryopreservation did not affect the activity of γGT and dipeptidases.

Since uptake of xenobiotics via organic anion transporters is another determinant of biotransformation capacity of RPT cells, we studied organic anion uptake of both type of slices using para-aminohippuric (PAH) acid as model compound. Compared with freshly isolated slices, cryopreserved rat renal cortical slices exhibited similar PAH uptake characteristics.

Concluding remarks

Selective exposure of confluent monolyers of rat renal proximal tubular cells to glutathione conjugates of the tested quinone and hydroquinones revealed that these compounds were more toxic after basolateral exposure than after apical exposure. In addition, the observed cytotoxicity appeared to be the result of mainly extracellular redox cycling of tested (hydro)quinones. The physiological significance of the observed effects remains obscure since our *in vitro* model suffers from several artefacts.

For instance, we could not observe basolateral uptake of GSH conjugated menadione and ethacrynic acid. In addition, the results obtained with GSH conjugated 2,5-difluoronitrobenzene (F*G*NB) suggest that apical uptake of cysteine-*S*-conjugates is also absent in our RPT cell monolayer. Probably as a result of the fact that the latter characteristic is a prerequisite for *N*-acetylation of these cysteine-*S*-conjugates by RPT cells, we were not able to measure *N*-acetylation of these conjugates. As a consequence of non-functional cysteine transport, large amounts of (hydro)quinone-thioethers remain outside the RPT cell. Since hydroquinones readily autoxidize under culture conditions, cytotoxicity emerges as a result of extracellular redox cycling.

In contrast to cultured RPT cells, the *N*-acetylation capacity was present in freshly isolated RPT cells. In another *in vitro* model it was shown that freshly isolated rat renal cortical slices also metabolised F*G*NB into its *N*-acetylcysteine conjugate. However, cryopreservation of rat renal cortical slices affected its *N*-acetylation capacity. Similar effects of cryopreservation were observed for organic anion transport by renal cortical slices.

In summary, RPT cell monolayers are useful for studying γGT and dipeptidase mediated

metabolism of glutathione conjugates. With respect to metabolic capacity, freshly isolated renal cortical slices provide a more appropriate tool for investigating xenobiotic metabolism *in vitro*. However, the results obtained from both *in vitro* models underline the necessity to characterise renal biotransformation and transport systems involved in renal metabolism of xenobiotics. In addition, functional limitations of the *in vitro* model used have to be discerned. In order to improve the usability of RPT cell cultures it is of paramount importance to evaluate the influence of culture environment on the expression of differentiated cellular characteristics.

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CHAPTER 8

Samenvatting en slotbeschouwingen

Samenvatting

Renale proximale tubulus (RPT) cellen kunnen aan apicale en basolaterale zijde worden blootgesteld aan, respectievelijk, glomerulair gefiltreerde en niet gefiltreerde niertoxische verbindingen. Om deze componenten uit te scheiden via de urine zijn de RPT cellen uitgerust met transport systemen die de niertoxische verbindingen van de basolaterale zijde naar de apicale zijde van de tubulus transporteren. Omdat niertoxiciteit tot dusver voornamelijk bestudeerd is in *in vitro* modellen zoals suspensies van RPT cellen of RPT cellen gekweekt op een vaste ondergrond, waarin transportsystemen slechts gedeeltelijk of niet meer functioneel zijn, is dan ook weinig aandacht besteed aan de rol van transport systemen in niertoxiciteit. Een adequaat *in vitro* systeem voor de bepaling van de toxiciteit voor RPT cellen van niertoxische verbindingen zou moeten beschikken over functionele transport systemen. Het primaire doel van dit proefschrift was dan ook het ontwikkelen van een dergelijk model. RPT cellen van rattenieren werden gekweekt tot een aaneengesloten (confluente) cellaag (monolayer) op een poreuze ondergrond. Deze monolayer bleef confluent gedurende 24 uur waarna het aaneengesloten karakter verloren ging en de viabiliteit van de cellen langzaam afnam (Hoofdstuk 2).

Als model verbindingen zijn glutathion (GSH) conjugaten van (hydro) chinonen, nitrobenzeen en ethacrynezuur gebruikt. Deze verbindingen kunnen *in vivo* door RPT cellen worden opgenomen omdat deze cellen uitgerust zijn met zowel enzym als transportsystemen die deze potentiëel toxische verbindingen als substraat kunnen gebruiken.

Het GSH conjugaat van menadion (MGNQ) was alleen toxisch na basolaterale blootstelling van confluente monolayers van RPT cellen (Hoofdstuk 2). Een toename in de MGNQ-geïnduceerde toxiciteit trad op wanneer de cellulaire opname van MGNQ via organisch anion transportsystemen werd geremd. Een toename in de toxiciteit werd ook waargenomen na inhibitie van de basolaterale opname van M(NAC)NQ, het *N*-acetylcysteine conjugaat van menadion, en na inhibitie van de deacetylering van M(NAC)NQ. Algemeen wordt aangenomen dat GSH conjugaten door RPT cellen worden opgenomen na metabolisme van deze conjugaten m.b.v. γ-glutamyltranspeptidase (γGT) en dipeptidase in cysteine conjugaten. MGNQ veroorzaakte alleen celtoxiciteit na remming van γGT. Deze resultaten suggereren dat cellulaire opname van thioether conjugaten (MGNQ, MNACNQ) geassocieerd is met detoxificatie van deze verbindingen. Het mogelijk hierbij betrokken reactie mechanisme dat resulteert in detoxificatie van het GSH en *N*-acetylcysteine conjugaat van menadion is weergegeven in Fig. 4 van

Hoofdstuk 2.

Detoxificatie van chinon cystein(glycinyl) conjugaten kon plaatsvinden via een intramoleculaire cyclisatie reactie waarmee de reactieve chinon functie verloren ging (Monks *et al.*, 1990). De identificatie en karakterisering van het cyclisatie product van MGNQ leverde een sterke aanwijzing dat deze reactie verantwoordelijk zou kunnen zijn voor de waargenomen detoxificatie van MGNQ door RPT cellen in dit *in vitro* systeem (Hoofdstuk 4).

Net als MGNQ waren beide mono-gesubstitueerde GSH conjugaten van 2-*tert*-butyl hydrochinon (5SG-TBHQ en 6SG-TBHQ) alleen toxisch na basolaterale blootstelling van een confluente monolayer van RPT cellen (Hoofdstuk 3). Blijkbaar is de γGT-gemediëerde omzetting van GSH conjugaten aan deze zijde van de cellaag niet voldoende voor complete detoxificatie van deze conjugaten in dit *in vitro* systeem (Hoofdstukken 2 en 3). Het di-gesubstitueerde GSH conjugaat van TBHQ (3,6SG-TBHQ) was niet toxisch in ons systeem. In overeenstemming met dit effect werd door Van Ommen *et al.* (1992) waargenomen dat 3,6SG-TBHQ minder reactieve zuurstof species kan genereren vergeleken met beide mono-gesubstitueerde conjugaten, hetgeen suggereert dat de (hydro)chinon-geïnduceerde RPT cel toxiciteit voornamelijk het gevolg is van "redox cycling" (Hoofdstuk 3). Superoxide dismutase en katalase elimineerden de SG-TBHQ geïnduceerde celtoxiciteit. Omdat beide verbindingen niet de cel in getransporteerd kunnen worden, lijkt het erop dat de chinon-thioether-geïnduceerde "redox cycling" voornamelijk extracellulair plaatsvindt (Hoofdstukken 2 en 3).

Apicale blootstelling van RPT cellen gekweekt op een vaste ondergrond aan 5SG-TBHQ resulteerde in celtoxiciteit terwijl 6SG-TBHQ geen toxiciteit kon induceren. Er werd geen celtoxiciteit ten gevolge van 5SG-TBHQ waargenomen na inhibitie van γGT met behulp van acivicin. In tegenstelling hiermee had inhibitie van γGT wel een cytotoxisch effect van 6SG-TBHQ in RPT cellen tot gevolg. Dit resultaat onderstreept de opvatting dat γGT aan de ene kant een detoxificatie kan initiëren van GSH geconjugeerde (hydro)chinonen door de reactieve chinon-functie van 5SG-TBHQ te verwijderen. Aan de andere kant kan γGT GSH conjugaten omzetten in metabolieten die gemakkelijker geoxideerd kunnen worden (6SG-TBHQ) (Hoofdstuk 3).

Er kon geen transepitheliaal transport van MGNQ en/of metabolieten ervan worden waargenomen over de monolayer van RPT cellen. Transport van deze verbindingen vindt hoogstwaarschijnlijk plaats als gevolg van paracellulaire lekkage hetgeen mogelijk het resultaat is van een afname in monolayer integriteit tengevolge van extensieve "redox cycling" van het chinon tijdens kweekcondities. Er werd tevens geen basolateraal naar apicaal transport waargenomen van het GSH conjugaat van ethacrynezuur (een α,β unverzadigd keton: EASG) over de monolayer van RPT cellen. Het is mogelijk dat de transporter die verantwoordelijk is voor het transport van dit conjugaat zijn functie heeft verloren in dit model. In tegenstelling hiermee werd alleen apicaal naar basolateraal transport van EASG waargenomen. Omdat acivicin middels de remming van γGT zowel het apicaal naar basolateraal transport van EASG inhibeerde

alsmede de vorming van vrij, niet-geconjugeerd EA, lijkt het erop dat niet het GSH conjugaat getransporteerd werd maar eerder het cysteine conjugaat en/of vrij EA (Hoofdstuk 4).

In Hoofdstuk 5 werd met behulp van ^{19}F-NMR waargenomen dat monolayers van LLCPK$_1$ cellen en primaire RPT cellen niet in staat waren het GSH conjugaat van 2,5-difluoronitrobenzeen (F*G*NB) te metaboliseren in het mercaptuurzuur (F*N*AcNB). Als gevolg hiervan was het cysteine conjugaat van 2,5-difluoronitrobenzeen (F*Cys*NB) het belangrijkste gevormde metaboliet. Vers geïsoleerde RPT cellen daarentegen waren in staat om mercaptuurzuren te vormen. Echter, direct na het in kweek brengen van deze cellen ging deze eigenschap verloren. Tevens werd geen apicaal naar basolateraal transport van F*Cys*NB waargenomen over monolayers van RPT cellen (Hoofdstuk 5).

In Hoofdstuk 6 werden vers geïsoleerde corticale slices van de rattenier getest op de mate waarin deze cellen toxische verbindingen (xenobiotica) metaboliseren. Dit werd gemeten aan de hand van de *N*-acetyleringscapaciteit van dit *in vitro* proximaal tubulair cel systeem. Er werd waargenomen dat deze slices F*Cys*NB metaboliseren in het mercaptuurzuur F*N*AcNB. Om na te gaan of cryo-gepreserveerde corticale nierslices als alternatief kunnen fungeren voor vers geïsoleerde slices in metabolisme studies, werd de biotransformatie capaciteit van beide typen slices vergeleken. De *N*-acetylerings capaciteit bleek vergelijkbaar voor ongeveer 4 uur maar nam daarna af in cryo-gepreserveerd weefsel. Cryopreservatie had geen effect op de activiteit van γGT en dipeptidases, getuige het feit dat de hoeveelheid F*Cys*NB gevormd door cryo-gepreserveerde slices uit F*G*NB niet lager was dan in vers geïsoleerd weefsel.

Opname van xenobiotica via organisch anion transporters kan een belangrijke determinant zijn van de biotransformatie capaciteit van RPT cellen. Immers, veel xenobiotica worden door RPT cellen opgenomen via deze transport systemen alvorens metabolisme van deze verbindingen kan plaatsvinden. Daarom werd de opname van organisch anionen door RPT cellen bestudeerd in beide typen slices waarbij gebruik werd gemaakt van para-aminohippuur (PAH) zuur als model verbinding. Vergeleken met vers geïsoleerde slices vertoonden cryo-gepreserveerde slices vergelijkbare PAH opname karakteristieken.

Slotbeschouwingen

Selectieve blootstelling van confluente monolayers van proximale tubulus cellen van de rattenier aan glutathion conjugaten van de geteste chinon en hydrochinonen laat zien dat deze verbindingen meer toxiciteit induceerden na basolaterale dan na apicale blootstelling van deze monolayers. Daarnaast bleek de waargenomen cytotoxiciteit voornamelijk het gevolg te zijn van extracellulaire "redox cycling" van de geteste (hydro)chinonen. Echter, de fysiologische relevantie van de waargenomen effecten blijft onduidelijk omdat een aantal artefacten werden waargenomen in ons *in vitro* systeem.

Er werd geen basolaterale opname van GSH geconjugeerd menadion en ethacrynezuur waargenomen. Daarnaast bleek uit resultaten verkregen uit transport studies met FGNB dat monolayers van RPT cellen waarschijnlijk ook geen apicale opname van cysteine conjugaten vertoonden. Mogelijk tengevolge van het feit dat laatstgenoemde eigenschap noodzakelijk is voor *N*-acetylering van cysteine-conjugaten werd ook geen *N*-acetylering van deze conjugaten waargenomen. Als gevolg van een niet-functionele cysteine transporter verblijven relatief hoge concentraties van (hydro)chinon-thioethers buiten de cel. Cytotoxiciteit als gevolg van extracellulaire "redox cycling" werd waargenomen hetgeen het gevolg was van het feit dat hydrochinonen vrij gemakkelijk auto-oxidatie kunnen ondergaan onder kweekcondities.

In tegenstelling tot gekweekte RPT cellen werd *N*-acetylering waargenomen in zowel vers geïsoleerde RPT cellen als in corticale slices van de rattenier. Echter, de *N*-acetyleringscapaciteit nam af na cryopreservatie van deze slices. Vergelijkbare effecten van cryopreservatie werden waargenomen op organisch anion transport door corticale slices van rattenieren.

Samenvattend kan gesteld worden dat monolayers van RPT cellen te gebruiken zijn voor γGT en dipeptidase-gemediëerd metabolisme van glutathion conjugaten. Gelet op de metabole capaciteit kan gesteld worden dat vers geïsoleerde corticale slices van de rattenier een bruikbaarder model vormen dan de monolayers van RPT cellen in de bestudering van het metabolisme van xenobiotica. De resultaten verkregen uit beide *in vitro* modellen onderstrepen de noodzaak tot karakterisering van renale biotransformatie en transport systemen die betrokken zijn in renaal metabolisme van xenobiotica. Daarnaast moeten de functionele tekortkomingen van een *in vitro* model goed onderkend worden. Om de bruikbaarheid van RPT cel cultures te vergroten is het van groot belang de invloed van kweekcondities op de expressie van gedifferentiëerde cellulaire functies te evalueren.

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CURRICULUM VITAE

Bert Haenen werd geboren op 8 december 1960 te Eijsden. In 1977 behaalde hij het MAVO diploma aan de plaatselijke MAVO "Serviam". Twee jaar later behaalde hij het HAVO diploma aan HAVO "Oud Vroenhoven" te Maastricht. In datzelfde jaar begon hij zijn studie aan de Nieuwe Leraren Opleiding (NLO) te Tilburg. Deze studie werd in 1984 met succes afgesloten met het behalen van een 2^e graads lesbevoegdheid in Biologie en een 3^e graads lesbevoegdheid in Scheikunde. In 1984 begon hij de doctoraal studie Biologie (Oud Curriculum) aan de Katholieke Universiteit te Nijmegen (biochemische oriëntatie). Het doctoraal examen werd in 1989 afgelegd met als hoofdvak Dierfysiologie (Dr. P.H.M. Balm, Dr. G. Flik en Prof. dr. S.E. Wendelaar Bonga) en de bijvakken Biochemie (Dr. P.H.G.M. Willems en Prof. dr. J.J.H.H.M. de Pont) en Algemene Heelkunde (dr. W.A. Buurman). Tijdens de doctoraalstudie behaalde hij de bevoegdheid stralingsdeskundige 5b alsmede de 1^e graads onderwijsbevoegdheid Biologie. Van september 1989 t/m oktober 1990 werd hij in de gelegenheid gesteld onderzoek te verrichten bij de vakgroep Dierfysiologie. Van november 1990 t/m januari 1995 werd hij aangesteld als Assistent in Opleiding bij de Vakgroep Toxicologie van de Landbouw Universiteit te Wageningen waar het in dit proefschrift beschreven onderzoek werd verricht. Tijdens deze periode leverde hij een bijdrage aan het Toxicologie onderwijs en behaalde hij de aantekening proefdierdeskundige (ex. art. 9 van de Wet op de Dierproeven). Daarnaast heeft hij de Post-doctorale Opleiding Toxicologie gevolgd. Thans is hij werkzaam als wetenschappelijk onderzoeker dossierbeoordeling diergeneesmiddelen bij het Rijks Kwaliteits Instituut voor Land -en Tuinbouwproducten (RIKILT-DLO) te Wageningen.

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