DNA-BINDING OF SULFUR-CONTAINING METABOLITES FROM ^{35}S-(PENTACHLOROBUTADIENYL)-L-CYSTEINE IN BACTERIA AND ISOLATED RENAL TUBULAR CELLS

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CONTENTS

SUMMARY

S-(Pentachlorobutadienyl)-L-cysteine (PCBC) is the penultimate metabolite formed from the nephrocarcinogen hexachlorobutadiene (HCBD). It is activated by cysteine conjugate β-lyase (β-lyase) to yield thioacylating metabolites thought to be responsible for PCBC-induced cytotoxicity and mutagenicity. We have investigated the β-lyase dependent DNA-binding of metabolites formed from ^{35}S-PCBC in Salmonella typhimurium (S. typhimurium) TA100 and in rat renal proximal tubule cells. ^{35}Sulfur was found in DNA isolated from S. typhimurium (410 ± 93 DNA-adducts per 10^6 nucleotides) and renal cells (68 or 97 DNA-adducts per 10^6 nucleotides). Enzymatic hydrolysis of the isolated DNA to yield 3'-nucleotide phosphates and fractionation of the hydrolysate by HPLC indicated the presence of 3 distinct, ^{35}S-containing metabolites which did not coelute with unchanged 3'-nucleotide phosphates and likely represent DNA constituents modified by ^{35}S-PCBC metabolites. Identical retention volumes were obtained for altered bases isolated from bacteria and from renal cells. The results obtained indicate that PCBC metabolites may covalently bind to DNA and implicate genotoxic mechanisms in HCBD-induced nephrocarcinogenicity.

I. INTRODUCTION

Hexachloro-1,3-butadiene (HCBD) is highly nephrotoxic in rats and induces renal tumors after long term application of nephrotoxic doses /1/. The molecular mechanism of HCBD-bioactivation has recently been elucidated /12/. HCBD is activated by conjugation with glutathione (GSH): the GSH S-conjugate formed, S-(pentachlorobutadienyl)glutathione, or S-conjugates formed by enzymatic peptide hydrolysis of the GSH S-conjugate are concentrated in the kidney. The cysteine S-conjugate S-(pentachlorobutadienyl)-L-cysteine (PCBC) is a substrate for renal cysteine conjugate β-lyase /3/ and is cleaved to yield reactive intermediates, a thioketene or a thioacylchloride /4/. Covalent binding of these intermediates to critical macromolecules in renal tubule epithelial cells is presumed to result in toxicity and may initiate tumor formation.

However, the molecular mechanism of tumor initiation is still

unclear. PCBC, whose metabolism by β-lyase is thought to be responsible for HCBD-induced toxicity and carcinogenicity, is a potent mutagen in bacteria /3, 5/ and genotoxic in renal cells in culture /6/. It is also a potent mitochondrial toxin and highly cytotoxic /7/. These factors indicate that both repeated toxic damage and irreversible DNA-binding may be critical events in HCBD-induced carcinogenesis.

In the experiments reported here, the DNA-binding of metabolites formed from ^{35}S-PCBC was measured in bacteria and in rat renal tubule epithelial cells in order to obtain further information on the mechanisms of HCBD-nephrocarcinogenicity.

II. MATERIALS AND METHODS

2.1 Syntheses

^{35}S-(pentachlorobutadienyl)-L-cysteine was synthesized by the reaction of ^{35}S-L-cysteine (specific activity 84 mCi/mmol. New England Nuclear, Dreieich, F.R.G.) with HCBD in liquid ammonia. The PCBC formed was isolated by prep. HPLC (for methods, see reference 5) and its identity was confirmed by UV-spectrometry and GC/MS after derivatisation /4/.

2.2 Modification and isolation of bacterial DNA

Overnight cultures of S. typhimurium TA100 (the strain was kindly provided by Prof. B.N. Ames) were washed and resuspended in 0.1 M sodium phosphate buffer (pH 7.4). 25ml of the cell suspension (3×10^{10} cells/ml as determined by optical density measurement at 595 nm) were then exposed for 1h to 50μM ^{35}S-PCBC dissolved in 50μl MeOH. After centrifuging and washing the cells with 0.15 M sodium chloride (pH 8) containing 0.1 M EDTA, bacterial DNA was isolated using a modification of the procedure described by Marmur /8/. Briefly, the cells were incubated with lysozyme for 25 min, frozen in liquid nitrogen and then lyzed with 0.1 M Tris buffer (pH 9) containing 1% sodium dodecyl sulfate (SDS). Deproteinisation was subsequently performed by treatment with phenol and subsequent extraction by chloroform-

isoamylalcohol (24:1) followed by precipitation of the DNA with 96% ethanol supplemented with 2M LiCl (12h at −20°C). The precipitated DNA was dissolved in 0.1 x SSC (15 mM NaCl and 1.5 mM sodium citrate) and RNA was removed by treatment with RNAse (Sigma, St. Louis, MO., USA) for 35 min. The deproteinisation - RNAse procedure was performed twice and the DNA was finally dissolved in 0.01 x SSC buffer.

2.3 Modification and isolation of renal DNA

Rat kidney proximal tubule cells were isolated from male Wistar rats (Institut für Versuchstierkunde, Hannover, F.R.G.) weighing 200-300 g. using the collagenase perfusion method of Ormstad et al. /9/. Cell yield and viability were determined in the presence of 0.2% trypan blue. Typically 85-95% of the cells excluded trypan blue after isolation from the kidney and approximately 20×10^8 cells were obtained from one kidney. Cells (final density 1.2×10^6 cells/ml) were incubated with ^{35}S-PCBC (200 mM) for 1h in Krebs-Henseleit buffer (pH 7.4) supplemented with 25 mM Hepes, 2.5 mM $CaCl_2$, 15 mM $NaHCO_3$ and 2% bovine serum albumin. After incubation, the cells were washed twice and nuclear DNA was isolated using a modification of the method described by Blin and Stafford /10/. Treatment with RNAse, proteinase and SDS (2h at room temperature) was followed by phenol and chloroform/isoamyl-alcohol extraction. The procedure was performed twice and the DNA was finally precipitated overnight at −20°C with 3M sodium acetate (1:10, v:v) and 96% ethanol (2.5:1, v:v). The amount and purity of DNA in both bacterial and renal cell DNA preparations were analyzed by spectrophotometry (A_{260} and A_{260}:A_{280}).

2.4 Enzymatic hydrolysis and HPLC separation

After lyophilisation and redilution of the DNA samples (final concentration 1μgDNA/μl) in 0.01 x SSC buffer (pH 7.5) digestion was performed by incubation of 10μg DNA with 50 U micrococcal nuclease (Boehringer Mannheim GmbH, Mannheim, F.R.G.) and 0.1 U spleen phosphodiesterase (Sigma, St. Louis, MO., U.S.A.) in 50 mM sodium succinate/10 mM $CaCl_2$ buffer pH 6.0 (final incubation volume 100μl) for 35 min at 37°C.

The samples were then filtered through a 0.45 M filter and fractionated by HPLC using a Waters (Milford, USA) HPLC system consisting of two M6000A pumps, a model 660 gradient controller and an U6K injector coupled to a Hewlett-Packard 1040 Diode-Array detector. Separations were performed on a Supelcosil LC-18S column (250 x 40 mm) using the following conditions: 10 min isocratic 50 mM sodium acetate (pH 5.3), then a linear gradient from 0-100% CH_3CN in 50 min: flow rate 1 ml/min. The eluate was collected into liquid scintillation vials and radioactivity was determined by liquid scintillation spectrometry (20 min counting time per sample). The eluate was also monitored by UV-spectroscopy at 254 nm.

III. RESULTS AND DISCUSSION

The potent mutagenicity of PCBC in S. typhimurium /3,5/ suggests that bacteria are an appropriate system for studies of binding of reactive sulfur containing intermediates derived from PCBC to DNA. S. typhimurium possess high β-lyase activity and can therefore cleave haloalkenyl cysteine S-conjugates efficiently /5/: moreover, bacterial DNA is more readily accessible to electrophiles than the mammalian genome. Also, the interference of PCBC with mitochondrial function /7/ which may prevent or mask genotoxicity does not occur in bacteria. There are, however, significant differences between bacteria and kidney tubule cells, the *in vivo* targets of cysteine conjugates derived from halogenated alkenes, differences not only in activation and detoxication reactions but also in transport systems and DNA repair capabilities. We therefore investigated the DNA-binding of PCBC both in bacteria and in primary proximal tubule cells isolated from the rat kidney.

DNA isolated from S. typhimurium incubated with 50 mM ^{35}S-PCBC for 1 h was free from protein contamination according to determination of A_{260}:A_{280} From the radioactivity determined per mg isolated DNA and the specific radioactivity of the ^{35}S-PCBC used, 410 ± 93 DNA-adducts per 10^8 nucleotides (mean ± S.D. from 3 independent experiments) were estimated. Enzymatic hydrolysis of the isolated DNA to yield 3'-nucleotide phosphates and fractionation of the hydrolysate by HPLC indicated the presence of 3 distinct, ^{35}S-containing metabolites which did not coelute with unchanged 3'-nucleotide phosphates nor with other UV absorbing

peaks and presumably represent DNA constituents modified by reactive, sulfur retaining intermediates derived from ^{35}S-PCBC (Fig. 1). The UV-chromatogram showed the presence of unchanged nucleotides and also small amounts of nucleosides which were identified by UV spectroscopy: formation of nucleosides could be accounted for by a low phosphatase activity of the spleen phosphodiesterase used.

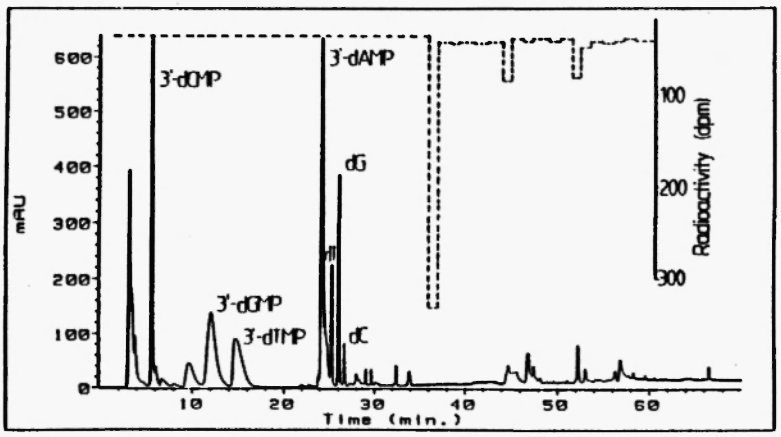

Fig. 1: HPLC-elution profile (— UV, 254 nm: — radioactivity) obtained from chromatographic separation of enzymatically hydrolysed DNA isolated from S. typhimurium TA 100 after treatment with 50μM ^{35}S-(pentachlorobutadienyl)-L-cysteine. For chromatographic conditions, see Materials and Methods.

Nuclear DNA isolated from rat kidney proximal tubule cells exposed to 200μM ^{35}S-PCBC for 1 h also contained radioactivity: adduct frequencies of 68 and 97 DNA-adducts per 10^6 nucleotides were found in two independent experiments. HPLC fractionation after hydrolysis revealed the presence of 3 radioactive peaks (Fig. 2) whose retention volumes were identical with those of the three ^{35}S-retaining metabolites obtained after hydrolysis of the bacterial DNA.

In both cases, the radioactive compounds present were not stable and hydrolysed slowly in aqueous solution releasing inorganic ^{35}sulfur (S^{--}, SO_4^{--}, HSO_3^-). The amounts of the ^{35}S-labelled DNA

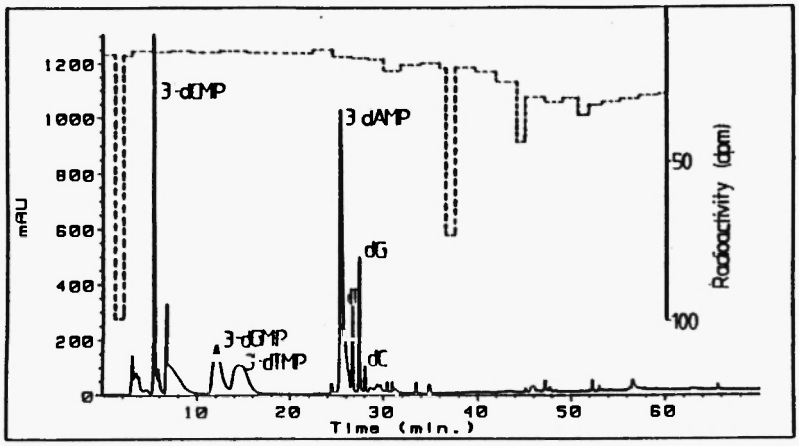

Fig. 2: HPLC-elution profile (— UV, 254 nm: — radioactivity) obtained from chromatographic separation of enzymatically hydrolysed DNA isolated from rat renal proximal tubule cells after treatment with 0.2 mM ^{35}S-(pentachlorobutadienyl)-L-cysteine. For chromatographic conditions, see Materials and Methods.

bases isolated in these experiments were insufficient for structural analysis. PCBC 1 (Fig. 3) is transformed by β-lyase to the enethiol 2 which is unstable and readily converted to acylating agents. The reactive metabolite(s) generated from PCBC react with the model nucleophile diethylamine to yield 2,3,4,4-tetrachlorothiobut-3-enoic acid diethylamide /4/ suggesting that the intermediates formed are thioacylating agents, presumably tetrachlorothiobutenoic acid chloride 3 or (1,1,2-trichlorovinyl)-1-chlorothioketene 4. Both thioacylchlorides and thioketenes may react with nitrogen nucleophiles to yield thioamides /11/. Based on the known reactivity of these intermediates an acylation of nitrogen atoms in DNA-bases (e.g. N-2 in guanosine) is conceivable (Fig. 3). The thioamide formed may not be stable in aqueous solution and hydrolyze slowly to the corresponding amide /12/. This known spontaneous hydrolysis is in accordance with the observed instability of the ^{35}S-containing adducts; however, the promutagenic DNA-lesions are only modified, not eliminated by this hydrolysis, since the resulting amides are stable at physiological pH. Radioactivity in DNA was certainly not due to incorporation of ^{35}S into the bases, because i) sulfur is not

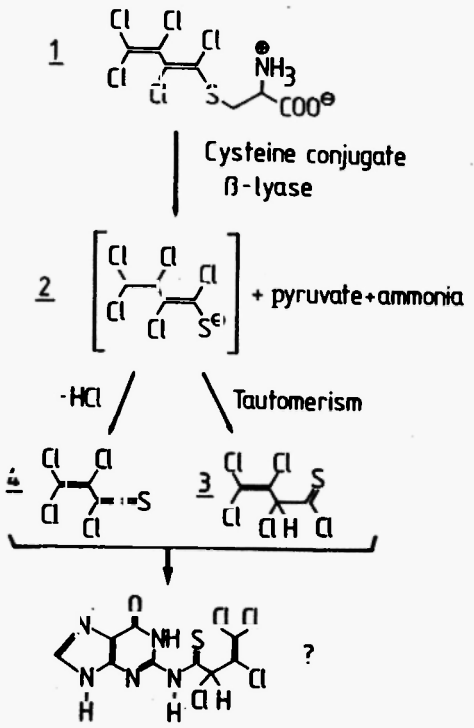

Fig. 3: Schematic of -Lyase mediated cleavage of S-(pentachlorobutadienyl)-L-cysteine to genotoxic intermediates.

incorporated into DNA and ii) unmodified bases and radioactive peaks had clearly different retention volumes in the HPLC separations. The results obtained with ^{35}S-PCBC in bacteria and renal cells correlate well with previous reports on the DNA-binding of reactive intermediates formed from ^{35}S-DCVC /13, 14/. Incubation of ^{35}S-DCVC with DNA in the presence of a partially purified β -lyase preparation resulted in alterations of the physico-chemical properties of the DNA and in the covalent binding of ^{35}S-containing metabolites to DNA. These authors also observed a slow hydrolysis of the ^{35}S from DNA after enzymatic DNA-hydrolysis /13,14/.

The results described demonstrate the ability of the HCBD metabolite PCBC to interact with DNA and support the concept that β-lyase cleavage of haloalkenylcysteine conjugates to reactive inter-

mediates initiate alteration of the renal DNA in vivo and thus be responsible for the nephrocarcinogenic activity of the parent haloalkenes. Further investigations to elucidate the structure of the DNA-adducts and their persistence in the kidney are required to confirm their role in the observed organ-, species- and sex-specific carcinogenic potential of HCBD.

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