Induction of glutathione *S*-transferase P-form in primary cultured rat liver parenchymal cells by co-planar polychlorinated biphenyl congeners

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Alterations in protein synthesis in primary cultured rat liver parenchymal cells were examined after their exposure to the potent carcinogens, polychlorinated biphenyl (PCB) congeners. Co-planar PCB congeners (3,4,5,3',4'-PCB and 3,4,5,3',4',5'-PCB) (10 nM) induced a protein, the M_r of which was 25000 (25 k protein) under denaturing conditions. However, non-co-planar PCB congeners and several xenobiotics, which induce microsomal proteins, did not induce the 25 k protein. By using immunoblotting, the 25 k protein was identified as glutathione *S*-transferase P-form (GST-P, 7–7, EC 2.5.1.18).

INTRODUCTION

Polychlorinated biphenyl (PCB) congeners and related chemicals are potent hepatocarcinogens, which from experimental evidence and epidemiological studies are suspected of promoting tumours [1–3]. These compounds also cause several other adverse effects, such as acne. Their toxicity is known to depend on the structure of the congeners [4,5]. Generally, co-planar PCB congeners are more toxic than non-co-planar PCBs. Co-planar congeners are chlorinated at the *meta* and *para* positions, whereas the non-co-planar congeners are chlorinated at *ortho* positions. The carcinogenic mechanism of PCB congeners has yet to be revealed, and their structure–activity relationship has not been well explained.

Co-planar PCB congeners are already known to be efficient inducers of cytochromes *P*-450IA1 and *P*-450IA2, as in the case of methylcholanthrene, and to enhance related drug-metabolizing enzyme activities in liver cells [5,6]. However, the carcinogenicity and toxicity of PCB congeners cannot be explained simply by the induction of cytochrome *P*-450s. Although alterations in the expression of genes other than those encoding cytochrome *P*-450s are thought to be involved in the hepatocarcinogenesis caused by PCB congeners, the genes induced by PCB congeners have not been systematically surveyed.

We aimed to identify the proteins induced by PCB congeners in liver cells. Primary cultured rat liver parenchymal cells were exposed to co-planar and non-co-planar PCB congeners. Co-planar PCB congeners (3,4,5,3',4'-PCB and 3,4,5,3',4',5'-PCB) increased the expression of a protein, the M_r of which was 25000 under denaturing conditions. This protein was identified immunochemically as glutathione *S*-transferase P-form (GST-P, 7–7, EC 2.5.1.18) [7–10] using the Western blotting technique.

Glutathione *S*-transferases are a family of enzymes that catalyse the conjugation of glutathione to various xenobiotics [11,12]. GST-P is normally expressed in tissues such as the placenta, lung and spleen, but not significantly in the liver [10]. However, it was detectable in preneoplastic hepatic foci and hepatocarcinomas caused by administration of chemical carcino-

gens [7–9], and is used as a pathological marker for these lesions [13,14]. We report here that GST-P was inducible in normal primary cultured liver parenchymal cells simply by exposing them to co-planar PCB congeners.

MATERIALS AND METHODS

Materials

Rat GST-P and rabbit IgG against rat GST-P were purified as previously reported [9]. 2,4,5,2',4',5'-PCB was purchased from GL Industries (Tokyo, Japan) and the other PCB congeners were kindly given by Dr. M. Morita (NIES, Tsukuba, Japan). Methylated ¹⁴C-labelled proteins were purchased from Amersham.

Preparation of monolayer cultured rat liver parenchymal cells

The liver of a male Wistar rat (about 150 g body weight) was perfused with a solution of collagenase *in situ* and liver parenchymal cells were isolated from the digested liver by the method of Seglen [15]. The viability of the isolated cells was determined by Trypan Blue exclusion as described previously [16]. The viability of the cells was over 90%. The isolated cells (0.85×10^6 cells) were plated in plastic 6-well plates (35 mm-diameter wells, Becton Dickinson, Lincoln Park, NJ, U.S.A.) with 1.5 ml of culture medium [William's E medium (Sigma) containing 10 mM-sodium pyruvate, 100 units of penicillin/ml, 100 µg of streptomycin/ml, 0.25 µg of fungizone/ml and 25 mM-Hepes/NaOH buffer, pH 7.4] containing 1 µM-insulin and 10% (v/v) fetal-calf serum (FCS; M.A. Bioproducts, Walkersville, MD, U.S.A.). These cells were cultured in a humidified incubator at 37 °C under CO₂/air (1:19).

The plastic plates were precoated with 0.15 mg of collagen/ml (Cellmatrix type I-P; Nitta Gelatin, Osaka, Japan) in Dulbecco's modified minimum essential medium (Gibco, Grand Island, NY, U.S.A.) for 5 h at 37 °C and then kept at 4 °C until use. The medium was changed 4 h after plating to remove non-adhered cells.

Abbreviations used: 25 k protein, a protein with an M_r of 25000; FCS, fetal calf serum; GST-P, glutathione *S*-transferase P-form (7–7, EC 2.5.1.18); NEPHGE, non-equilibrium pH-gradient electrophoresis; PCB, polychlorinated biphenyl; TBS, 20 mM-Tris/HCl buffer, pH 7.5 (25 °C) containing 0.5 M-NaCl; TTBS, 20 mM-Tris/HCl buffer, pH 7.5 (25 °C) containing 0.5 M-NaCl and 0.05% Tween-20.

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Exposure of the cells to chemicals

After incubation for 20 h, the cells were exposed to PCBs or other xenobiotics by exchanging the medium with identical medium to which chemicals had been added at the indicated concentrations. The chemicals were dissolved in dimethyl sulphoxide and then added to the culture medium before treatment of the cells. Control cells were exposed to the medium containing 0.1 % dimethyl sulphoxide.

In some experiments, the liver cells were cultured without FCS. After inoculation of the cells into the culture medium with 1 nM-insulin and 10 % (v/v) FCS, the adhered cells were further incubated in the culture medium containing 1 nM-insulin, 10 nM-dexamethasone and 0.1 µg of aprotinin/ml. After 20 h of incubation, the medium was removed and replaced with the culture medium containing PCB, 6 mM-nicotinamide and 0.1 µg of aprotinin/ml.

Labelling of newly synthesized proteins with [³⁵S]methionine

After exposure to chemicals for 23 h or the indicated time, the cells were washed twice with Earle's balanced salt solution (Sigma) and incubated for 1 h in Eagle's minimum essential medium deficient in methionine (Sigma) containing 100 µCi of [³⁵S]methionine/ml (800 Ci/mmol; ICN, Costa Mesa, CA, U.S.A.), 10 mM-Hepes/NaOH buffer, pH 7.4, 10 % (v/v) FCS [dialysed against Dulbecco's phosphate-buffered saline (Flow Laboratories, Irvine, Scotland, U.K.)] and PCBs or other xenobiotics added at the indicated concentrations. When the cells were incubated without FCS, it was omitted from the medium containing [³⁵S]methionine. After being washed twice with Earle's balanced salt solution, the cells were lysed by adding 0.2 ml of a lysis solution (1 % Nonidet P40, 0.1 % SDS and 1 mM-phenylmethanesulphonyl fluoride). The lysate was centrifuged at 2000 g for 5 min. The resulting supernatants (cell lysates) were stored at -70 °C. Radioactivity in the trichloroacetic acid precipitate of the cell lysates was measured by the method of Caltabiano *et al.* [17].

PAGE

Conventional SDS/PAGE was performed by the method of Laemmli [18]. The cell lysate was mixed with reagents to give 125 mM-Tris/HCl buffer, pH 6.8 (25 °C), 2 % (v/v) 2-mercaptoethanol, 0.1 % SDS, 10 % (v/v) glycerol and 0.05 % Bromophenol Blue. Sample solutions were applied to slab gels (16 cm × 14 cm, 0.75 mm or 1.5 mm thick) with separating gels containing 11 % (w/v) acrylamide/bisacrylamide (37:1, w/w) or 15 % (w/v) acrylamide/bisacrylamide (55:1, w/w), and separated at 15 mA/gel. ³⁵S-labelled proteins separated on the gel were detected by fluorography. After electrophoresis, the gel was fixed in acetic acid/methanol/distilled water (1:3:6, by vol.), immersed in En³Hance (NEN, Boston, MA, U.S.A.) for 2 h and washed with distilled water. The dried gels were exposed to X-Omat AR film (Kodak, Rochester, NY, U.S.A.).

Two-dimensional gel electrophoresis was performed using non-equilibrium pH-gradient electrophoresis (NEPHGE) for the first dimension according to the method of O'Farrell *et al.* [19]. The samples were diluted to 10 µl with a lysis solution, mixed with 5.5 mg of urea, 0.8 µl of 2-mercaptoethanol and 0.8 µl of 40 % (w/v) Ampholine (pH 3.5–10; Pharmacia/LKB, Uppsala, Sweden) and applied to the top of the first-dimension gels (12 cm × 1.5 mm diameter) made of 9 M-urea and 2 % (w/v) Ampholine as previously reported [20]. Urea solution (5 µl; 4.5 M) containing 1 % Ampholine was loaded on to the samples. After NEPHGE for 4 h at 400 V, the first-dimension gels were removed from their tubes, and incubated in 63 mM-Tris/HCl buffer, pH 6.8 (25 °C) containing 2 % (v/v) 2-mercaptoethanol,

1 % SDS and 10 % (v/v) glycerol at room temperature for 20 min. The gels were loaded on SDS/11 % (w/v) polyacrylamide gels (1.5 mm thick) and fixed with a solution containing 1 % agarose gel, 125 mM-Tris/HCl buffer, pH 6.8 (25 °C) and 0.1 % SDS. The second-dimension electrophoresis was performed at 30 mA/gel.

Western blotting and immunochemical detection of rat GST-P

Western blotting was carried out by the method of Towbin *et al.* [21]. After the proteins in the cell lysates were separated by conventional SDS/PAGE (1.5 mm thick) or on two-dimensional gels, they were transferred electrophoretically to a nitrocellulose membrane (Bio-Rad, Richmond, CA, U.S.A.). Transfer of proteins was carried out at 35 V constant for 18 h at 4 °C in a transfer chamber (Bio-Rad; width between electrode; 3.5 cm) filled with an electrode buffer [(25 mM-Tris/190 mM-glycine, pH 8.5)/methanol, 4:1, v/v]. The membrane was washed with distilled water, dried and then directly exposed to X-ray film to detect ³⁵S-labelled proteins before immunochemical staining.

GST-P was identified by immunochemical staining with a Protein-A-colloidal gold conjugate and enhanced with silver staining according to the manufacturer's instructions (Bio-Rad). The membrane was blocked with 3 % (w/v) gelatin (Bio-Rad) in TBS buffer [20 mM-Tris/HCl buffer, pH 7.5 (25 °C) containing 0.5 M-NaCl] for more than 2 h. Then it was incubated for 10 h at room temperature in 0.14 mg of rabbit anti-(rat GST-P) IgG/ml dissolved in TTBS buffer (TBS with 0.05 % Tween-20) containing 1 % gelatin. After being washed twice with TTBS buffer for 5 min each, the membrane was incubated in a Protein A-colloidal gold conjugate solution (Bio-Rad) overnight at room temperature, then rinsed with TTBS buffer, followed by distilled water. If the staining was too weak, it was enhanced with silver stain. The rinsed membrane was soaked in 0.2 M-sodium citrate buffer, pH 3.7, for 5 min, and incubated in a developer (0.85 % hydroquinone/0.11 % silver lactate in 0.18 M-sodium citrate buffer, pH 3.7) for 5–10 min. Colour development was stopped by washing the membrane in a 10-fold dilution of Bio-Rad fixer.

RESULTS

Primary cultures of rat liver parenchymal cells were exposed to 10 and 100 nM-co-planar PCB congeners (3,4,5,3',4'-PCB and 3,4,5,3',4',5'-PCB) by incubating them in culture medium containing FCS and these PCB congeners. As shown in Fig. 1, the expression of a protein with *M_r* 25000 (25 k protein) was markedly increased after 23 h of treatment with the co-planar PCB congeners. 3,4,5,3',4'-PCB was more effective in increasing expression of the 25 k protein than 3,4,5,3',4',5'-PCB. The expression of the 25 k protein was increased by exposure to both co-planar PCB congeners for 11 h. However, its expression was not increased when treated with 3,4,5,3',4'-PCB at concentrations less than 1 nM (results not shown).

The cells were exposed to other PCB congeners and xenobiotics in order to examine the specificity of the increased expression of the 25 k protein. As shown in Fig. 2, 25 k protein expression was increased slightly by 100 nM-3,4,3',4'-PCB but was not increased by 100 nM-non-co-planar congeners (2,3,4,3',4'-PCB, 2,3,4,5,3',4'-PCB and 2,4,5,2',4',5'-PCB), 2 mM-phenobarbital and 20 µM-*p*-aminoacetophenone. Diethylnitrosoamine, dibromoethane and 1,1-bis-(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) also had no effect (results not shown).

Because the apparent *M_r* of GST-P (25000) is similar to that of the 25 k protein [7–9,22], and it is known to be a marker for preneoplastic and neoplastic lesions caused by administration of chemical carcinogens, the relationship between the 25 k protein and GST-P was examined by immunoblotting. The lysates from

Fig. 1. Induction of the 25 k protein in primary cultured rat liver parenchymal cells exposed to co-planar PCB congeners

The cells were incubated in culture medium with FCS and were exposed to 3,4,5,3',4'-PCB at 10 nM (lane 1) and 100 nM (lane 2) and 3,4,5,3',4',5'-PCB at 10 nM (lane 3) and 100 nM (lane 4) for 23 h. Control cells were cultured in the absence of PCB congeners (lane C). The newly synthesized proteins were labelled with [³⁵S]methionine for 1 h. The cell lysates (160 000 c.p.m.) were separated by SDS/PAGE (11% gel) and the labelled proteins were detected by fluorography. Lane M contains methylated ¹⁴C-labelled proteins used as *M_r* markers: 97, phosphorylase *b* (*M_r* 93 000); 69, BSA (*M_r* 69 000); 46, ovalbumin (*M_r* 46 000); 30, carbonic anhydrase (*M_r* 30 000); and 14, lysozyme (*M_r* 14 000). The arrowhead indicates the position of the 25 k protein.

Fig. 2. Comparative induction of the 25 k protein by co-planar and non-co-planar PCB congeners and xenobiotics

Rat liver parenchymal cells were incubated in culture medium containing FCS and were exposed for 17 h to the following PCBs, all at 100 nM: 3,4,5,3',4'-PCB (lane 1); 3,4,5,3',4',5'-PCB (lane 2); 3,4,3',4'-PCB (lane 3); 2,3,4,3',4'-PCB (lane 4); 2,3,4,5,3',4'-PCB (lane 5); 2,4,5,2',4',5'-PCB (lane 6). The proteins in lane 7 and 8 are from cells exposed for 17 h to 2 mm-phenobarbital and 20 μM-*p*-aminoacetophenone respectively, and the proteins in lane C are from cells cultured in the absence of xenobiotics. Newly synthesized proteins were labelled with [³⁵S]methionine for 1 h. The cell lysates (60 000 c.p.m.) were separated by SDS/PAGE (15% gel), and the labelled proteins were detected by fluorography. Lane M contains the same labelled proteins used as *M_r* markers in Fig. 1. The arrowhead indicates the position of the 25 k protein.

Fig. 3. Detection of GST-P induced in primary cultured rat liver parenchymal cells after exposure to 3,4,5,3',4'-PCB

The cells were cultured without FCS and exposed to 100 nM-3,4,5,3',4'-PCB for 23 h. The cell lysates (1000 000 c.p.m.) from cells exposed to PCB and control cells were analysed by SDS/PAGE (15% gel). ³⁵S-labelled proteins separated on the gel were transferred to a nitrocellulose membrane. The labelled proteins on the membrane were detected autoradiographically on X-ray film. Then GST-P was detected on the membrane immunochemically using rabbit anti-(rat GST-P) IgG and a Protein A-colloidal gold conjugate. Lanes 1 and 2, autoradiogram. Lanes 3, 4 and G, immunochemical detection of GST-P. Lanes 1 and 3, the cell lysate from the control cells. Lanes 2 and 4, the lysate from cells exposed to 3,4,5,3',4'-PCB. Lane G, purified rat GST-P (1 μg).

cells exposed to 100 nM-3,4,5,3',4'-PCB were separated by conventional SDS/PAGE, and transferred to a nitrocellulose membrane. The proteins immobilized on the membrane were detected by autoradiography and GST-P was stained immunochemically. As shown in Fig. 3, the band reacting to the anti-(GST-P) antibody was detected in the lysate of cells exposed to 3,4,5,3',4'-PCB. This band corresponded to the position of the 25 k protein and its *M_r* was the same as that of the authentic rat GST-P. GST-P was not detectable in the control lysate.

From these results, we assumed that the 25 k protein was identical with GST-P and that this enzyme was inducible by 3,4,5,3',4'-PCB in primary cultured rat liver parenchymal cells. In these experiments, the cells were cultured in the absence of FCS. However, when the cells were incubated in culture medium containing FCS, GST-P, which is not normally detectable in rat liver cells, appeared in the control cells which had not been exposed to PCB congeners (results not shown). Therefore FCS appears to contain a factor or factors that might stimulate the synthesis of GST-P. Nevertheless, GST-P which was induced by FCS in the control cells was not labelled with [³⁵S]methionine, indicating that GST-P is synthesized in these cells at a very low rate.

Induction of GST-P synthesis by 3,4,5,3',4'-PCB was confirmed by two-dimensional gel electrophoresis. In Fig. 4, an autoradiogram of the membrane shows that the 25 k protein identified in a one-dimensional gel separated into two proteins after two-dimensional gel electrophoresis. Immunochemical staining indicated that one of the 25 k proteins (an acidic one) bound the anti-(GST-P) antibody (Figs. 4a and 4b, the right circle). This 25 k protein also co-migrated with purified GST-P on a two-dimensional gel (results not shown), confirming that GST-P is induced by 3,4,5,3',4'-PCB. In addition to induction of the 25 k protein, 3,4,5,3',4'-PCB also increased the expression of

Fig. 4. Immunochemical identification of GST-P by two-dimensional gel electrophoresis of ^{35}S -labelled proteins from cells exposed to 3,4,5,3',4'-PCB

The cells were cultured without FCS and exposed to 100 nm-3,4,5,3',4'-PCB for 23 h. Lysates (330 000 c.p.m.) from cells exposed to PCB and control cells were subjected to two-dimensional gel electrophoresis using NEPHGE for the first dimension. ^{35}S -labelled proteins separated on the gel were transferred to a nitrocellulose membrane. After detection of the labelled proteins on the membrane by autoradiography, GST-P was detected immunochemically using rabbit anti-(rat GST-P) IgG and a Protein A-colloidal gold conjugate. The detection was enhanced by silver staining. (a) and (b) Cell lysate from cells exposed to PCB. (a) Immunochemical staining of GST-P; (b) Autoradiogram. (c) Autoradiogram of the lysate from control cells. The cell lysates shown in (a), (b) and (c) were subjected

two other proteins and caused several other changes in protein synthesis (Fig. 4). These increased proteins have not yet been identified.

DISCUSSION

The present study demonstrates that GST-P is inducible in primary cultured rat liver parenchymal cells after exposure of the cells to a co-planar PCB congener, 3,4,5,3',4'-PCB. Another co-planar PCB congener, 3,4,5,3',4',5'-PCB, also induced the 25 k protein (GST-P) efficiently, while 3,4,3',4'-PCB caused only slight induction. Non-co-planar PCB congeners did not induce the 25 k protein at all. Several xenobiotics, which are known to induce drug-metabolizing enzymes and microsomal proteins [23–28], were also ineffective in inducing GST-P within the exposure for 17 h. However, Vandenberghe *et al.* [29] reported that phenobarbital induced GST-P in primary cultured liver parenchymal cells after a prolonged incubation of over 2 days. These results suggest that GST-P is specifically induced by co-planar PCB congeners, and that induction of this enzyme is not due to a non-specific increase in microsomal protein synthesis caused by xenobiotics.

Co-planar PCB congeners are strong inducers of cytochromes *P*-450IA1 and *P*-450IA2 [4,6,24], and a mixture of PCB congeners is known to increase drug-metabolizing activity including glutathione *S*-transferase activity [26,30,31]. However, it was not known that co-planar PCB congeners alone could induce GST-P in normal liver parenchymal cells.

GST-P is a member of the glutathione *S*-transferase family that catalyses the formation of glutathione conjugates [32]. This enzyme is repressed in normal liver tissue, but is expressed specifically in preneoplastic and neoplastic cells after administration of chemicals to rats *in vivo* [13,14]. The induction of GST-P was also observed in rat liver after administration of Protein A [33]. Various organic anions are known to bind to glutathione *S*-transferase isoenzymes and inhibit the enzyme activity. Comparing it with other molecular forms of glutathione *S*-transferase, GST-P was shown to have broad substrate specificity and to be insensitive to the inhibitory actions of various organic anions such as bilirubin, haematin and bromosulphophthalein [34]. These characteristics of GST-P might be closely related to its induction in the neoplastic cells caused by chemical carcinogen.

Judging from the specific induction of GST-P by co-planar PCB congeners, GST-P may play a part in the detoxication of the chemicals, but the regulatory mechanism remains to be revealed. Co-planar PCB congeners are known to induce cytochrome *P*-450s by binding to an intracellular aryl-hydrocarbon ('Ah') receptor, and the PCB-aryl-hydrocarbon receptor complex associates with a specific responsible element in the genome [27,28]. Co-planar PCB congeners bind to the arylhydrocarbon receptor with higher affinity than non-co-planar congeners and induce cytochrome *P*-450s very efficiently. Because GST-P was induced by only co-planar PCB congeners in liver parenchymal cells, the aryl-hydrocarbon receptor may play a role in the induction of GST-P. However, it is unclear why GST-P and cytochrome *P*-450s are both expressed in liver parenchymal cells which are exposed to co-planar PCB congeners, although GST-P is not normally expressed in liver tissue, whereas cytochrome *P*-450s are. The possibility that GST-P induction observed in the

to SDS/PAGE shown at the right of each panel. Circles and arrowheads indicate the positions of the 25 k proteins. Squares indicate the proteins the expressions of which were increased in cells exposed to PCB. NEPHGE and SDS/PAGE indicate the direction of the first and second dimensions of electrophoresis respectively.

present study is a unique event confined to cultured cells cannot be ruled out.

The *cis*-acting regulatory DNA elements of GST-P have been analysed and multiregulatory elements have been identified. An upstream element responsible for phorbol ester was found in the 5'-flanking sequence of the GST-P gene [35]. Therefore it seems possible that processes independent of the aryl-hydrocarbon receptor could also participate in GST-P induction by co-planar PCB congeners [36,37].

Co-planar PCB congeners and related chemicals have been shown to cause a number of biological effects in addition to their carcinogenic activities. For example, polychlorinated dibenzodioxin and dibenzofuran alter the binding kinetics of epidermal-growth-factor receptor on the plasma membrane [38,39]. 3,4,5,3',4',5'-PCB was reported to act as a teratogen [40]. But all of the biological effects of co-planar PCB congeners cannot be explained simply by inductions of cytochrome P-450s. In order to understand various biological effects of co-planar PCB congeners and related chemicals, the cellular responses specifically induced by co-planar PCB congeners should be revealed. Further study on the process of GST-P induction in liver parenchymal cells is important for understanding the biological effects of co-planar PCB congeners.

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