Phosphorylation of c-Jun stimulated in primary cultured rat liver parenchymal cells by a coplanar polychlorinated biphenyl

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Phosphorylation of c-Jun was stimulated in primary cultured rat liver parenchymal cells by treatment with a coplanar polychlorinated biphenyl congener, 3,3′,4,4′,5-pentachlorobiphenyl (PenCB), as well as by epidermal growth factor, but was not stimulated by the non-coplanar form. However, the amount of c-Jun mRNA did not increase with PenCB treatment. PenCB may activate a signal-transducing pathway consisting of protein kinases.

INTRODUCTION

Coplanar polychlorinated biphenyl (PCB) congeners and related compounds, e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran, have been shown to be potent carcinogens in both experimental and epidemiological studies [1–4]. Coplanar PCB congeners are not strong mutagens and are categorized as tumour promoters. These compounds also exhibit teratogenicity [2] as well as other adverse characteristics, such as anti-oestrogen activity [5]. Coplanar PCB congeners are chlorinated at the *meta* and *para* positions, and their stereo-structures are similar to those of TCDD and 2,3,7,8-tetrachlorodibenzofuran. Congeners of this type incorporated into cells bind to an arylhydrocarbon-receptor (Ah-R). The ligand-bound Ah-R and auxiliary factors (e.g. Ah-R nuclear translocator protein) act together to express cytochrome *P*-450IA1 and related drug-metabolizing enzyme genes through interaction with specific DNA sequences (xenobiotic-responsive elements) on these genes [6,7]. However, the key reaction(s) by which these coplanar PCB congeners induce their various adverse effects has not yet been identified.

We showed previously that glutathione S-transferase (GST) P-form (GST-P; EC 2.5.1.18, Pi-class GST of rat) was induced in primary cultured rat liver parenchymal cells by treatment with 3,3′,4,4′,5-pentachlorobiphenyl (PenCB, an extremely toxic coplanar PCB congener) and was not induced by non-coplanar PCB congeners [8]. Pi-class GSTs are marker enzymes for preneoplastic hepatic foci but are latent in normal liver of humans and rodents [9,10]. The gene structure of GST-P and the mechanism for inducing GST-P in tumour cells have been intensively investigated [11]. The 5′-upstream region of the GST-P gene was shown to contain a phorbol 12-myristate 13-acetate (PMA)-responsive element (PRE) to which a nuclear transcription factor, AP-1 (the heterodimer of proto-oncogene products, c-Jun and c-Fos), or a related transcription factor (e.g. the homodimer of c-Jun) is able to bind [12]. These transcription factors may play a role in the expression of GST-P by a coplanar PCB congener. AP-1 activity is determined by the phosphorylation of c-Jun which is regulated by cell growth factors and environmental stressors through a cascade system of protein kinases (a signal-transducing pathway) [13–17]. In fact, GST-P mRNA was found to be expressed in liver parenchymal cells by treatment with epidermal growth factor (EGF) [18,19]. GST-P expression induced by PenCB, as well as by EGF, was reduced in the presence of protein kinase inhibitors and was also inhibited by dexamethasone [18], which is an antagonist of c-Jun activity [20–22]. These observations suggest that the phosphorylation of c-Jun is regulated by a coplanar PCB congener.

We report here that PenCB stimulated the phosphorylation of c-Jun expressed in primary cultured rat liver parenchymal cells, but that the amount of c-Jun mRNA did not increase. The phosphorylation of c-Jun may be a novel pathway for gene expression caused by a coplanar PCB congener.

EXPERIMENTAL

Materials

PenCB and pRJ101 [23] were kindly provided by Dr. M. Morita and Dr. M. Muramatsu respectively. The peptide epitope (a synthetic peptide, amino acids 209–225 of v-Jun [24] corresponding to 247–263 of rat c-Jun [23]) and an affinity-purified anti-c-Jun rabbit IgG raised against it were purchased from Oncogene Science (Manhasset, U.S.A.); 2,2′,4,4′,5,5′-hexachlorobiphenyl (HexaCB) was purchased from GL Industries (Tokyo, Japan); normal rabbit IgG, from Cappel (Durham, U.S.A.); Williams' E medium, from Sigma (St. Louis, MO, U.S.A.); Protein A–Sepharose 4B, from Pharmacia (Uppsala, Sweden); and ^{14}C-labelled proteins, from Amersham (Amersham, Bucks., U.K.).

Treatment of monolayer-cultured rat liver parenchymal cells with PenCB, HexaCB, PMA or EGF, and labelling of cellular proteins with radioisotopes

Liver parenchymal cells were isolated from a male Wistar rat liver after digestion with a collagenase solution as previously reported [18]. The isolated cells (0.25×10^6 cells) were incubated in collagen-coated 6-well plates (Corning Inc., Corning, U.S.A.) with 2 ml of modified Williams' E medium containing 10 % (v/v) fetal bovine serum. Incubation was carried out for 4 h in a 5 % CO_2 incubator at 37 °C and the medium was then changed to

Abbreviations used: Ah-R, arylhydrocarbon-receptor; EGF, epidermal growth factor; GST, glutathione S-transferase; GST-P, glutathione S-transferase P-form; HexaCB, 2,2′,4,4′,5,5′-hexachlorobiphenyl; MEM, Eagle's minimum essential medium; PCB, polychlorinated biphenyl; PenCB, 3,3′,4,4′,5-pentachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PMA, phorbol 12-myristate-13-acetate; PRE, PMA-responsive element.

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serum-free modified Williams' E medium [18]. The cells were incubated for an additional 20 h and then subjected to a 6 h or 30 min treatment with the reagents.

For the 6 h treatment, the medium was changed to 2 ml of serum-free modified Williams' E medium containing 6 mM nicotinamide and 100 ng/ml aprotinin [18], to which was added a one-hundredth volume of a DMSO solution of PenCB, HexaCB or PMA, or a solution of EGF to give a final concentration of 100 nM, 100 nM, 100 ng/ml or 10 ng/ml, respectively. An equal volume of DMSO was added to the control cell medium. After the cells were incubated for 3 h, they were washed twice with phosphate-deficient Eagle's minimum essential medium (MEM) [25] containing a single-strength non-essential amino acid mixture (Gibco, Grant Island, U.S.A.), the same vitamin mixture as in Williams' E medium, 10 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizon and 25 mM Hepes/NaOH buffer, pH 7.4. This supplemented medium was termed P(-)MEM. After washing, the medium was changed to 800 μl of P(-)MEM containing PenCB, HexaCB, PMA or EGF at the same concentration. After incubation for 30 min, sodium [^{32}P]orthophosphate (ICN, Costa Mesa, U.S.A.) was added to the culture at a concentration of 1 mCi/ml in order to label the proteins and the culture was continued for another 2.5 h.

For the 30 min treatment, cells were washed twice with P(-)MEM, and incubated in 800 μl of P(-)MEM for 30 min. [^{32}P]Orthophosphate was then added to the culture and the cells were incubated for another 2.5 h. A one-hundredth vol. of a solution of PenCB, HexaCB, PMA or EGF was added to the cultures to give the same final concentrations as indicated above for the final 30 min of cell labelling. For labelling with [^{35}S]methionine and [^{35}S]cysteine, the cells were washed twice with S(-)Williams' E medium which was methionine-, cysteine-, cystine- and glutathione-deficient Williams' E medium (Kyokuto, Tokyo, Japan) containing 10 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizon and 25 mM Hepes/NaOH buffer, pH 7.4, and incubated in 800 μl of this same medium for 30 min. EXPRE^{35}S^{35}S protein labelling mix (NEN, Boston, MA, U.S.A.) was added to the culture at concentrations of 0.25 mCi/ml [^{35}S]methionine and 0.1 mCi/ml [^{35}S]cysteine, and the cells were incubated for another 2.5 h. PenCB or EGF was added to the culture at the same final concentrations as indicated above for the final 30 min of cell labelling.

Immunoprecipitation with Protein A–Sepharose

All procedures were performed at 4 °C unless otherwise stated. ^{32}P- and ^{35}S-labelled cells were washed twice with P(-)MEM and S(-)Williams' E medium, respectively, and then lysed with RIPA buffer [26]. The lysates were clarified by centrifugation at 10000 ***g*** for 10 min. A sample (2 mg) of Protein A–Sepharose was blocked in IPP1 buffer (10 mM Tris/HCl buffer, pH 8.0, at 25 °C/500 mM NaCl/0.1 % Nonidet P-40) with an unlabelled lysate (400 μg of protein/ml) prepared from the cultures in which [^{32}P]orthophosphate had been omitted, by gently suspending it for 1 h and then washing twice with the same buffer. The blocked Protein A–Sepharose was mixed gently with 500 μl of IPP1 buffer containing 2.5 μg of anti-c-Jun rabbit IgG for 2 h, then washed twice, and re-mixed with radioisotope-labelled lysate (50 μg of protein) in 250 μl of IPP2 buffer (50 mM Tris/HCl buffer, pH 7.5, at 25 °C/150 mM NaCl/0.05 % Nonidet P-40). The radioisotope-labelled lysate had been previously mixed with 2 mg of normal rabbit IgG-immobilized Protein A–Sepharose for 1 h to remove proteins bound non-specifically to IgG and the resin before immunoprecipitation was performed. After washing four times with IPP2 buffer, proteins immobilized on the Protein A–Sepharose were solubilized in SDS-containing sample buffer, and then subjected to SDS/12.5 %-PAGE according to Laemmli [27]. The radioisotope-labelled proteins separated on the polyacrylamide gel were visualized by autoradiography using a bioimage analyser (BAS2000, Fuji Film, Tokyo, Japan), and the radioactivity of [^{32}P]orthophosphate incorporated into c-Jun was determined using the same bioimage analyser with its accompanying software (BAStation, Fuji Film). The protein concentration was determined using Bradford's method [28].

Extraction of RNA and determination of c-Jun mRNA

Total RNA was extracted from the cells cultured without [^{32}P]orthophosphate after treatment with PenCB, HexaCB, PMA or EGF for 6 h or 30 min, and was then separated on a formaldehyde/agarose gel [18]. The amount of c-Jun mRNA was determined by Northern blot analysis using an *Eco*RI fragment of pRJ101 as a cDNA probe [23].

RESULTS

Primary cultured rat liver parenchymal cells were treated with either 100 nM PenCB or 10 ng/ml EGF, by which GST-P is inducible [8,18], for 6 h and labelled with sodium [^{32}P]orthophosphate. c-Jun was isolated from the cell lysate by immunoprecipitation using anti-c-Jun IgG and a Protein A–Sepharose conjugate. c-Jun labelled with [^{32}P]orthophosphate was visualized by autoradiography after SDS/PAGE separation. As shown in Figure 1(a), it was detected as a retarded band at a position corresponding to a relative molecular mass (M_r) of about 45000. The amount of ^{32}P-labelled c-Jun had increased as a result of treatment with both PenCB and EGF, and the increase in the level of phosphorylation obtained with either treatment was similar. c-Jun was shown to be phosphorylated at several serine/threonine residues, which accounted for the retardation of the band on electrophoresis [14,24]. Several other proteins with M_r values of 50000–65000 were also phosphorylated after treatment with either PenCB or EGF. Some seemed to be

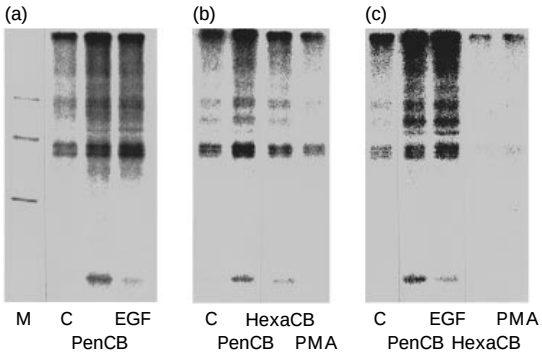

Figure 1 Stimulated phosphorylation of c-Jun by treatment with PenCB, EGF or other reagents for 6 h or 30 min

Primary cultured rat liver parenchymal cells were treated with the reagents for 6 h (**a** and **b**) or 30 min (**c**), and labelled with 1 mCi/ml [^{32}P]orthophosphate. After c-Jun was separated from the cell lysates by immunoprecipitation and subjected to SDS/PAGE, ^{32}P-labelled c-Jun was visualized by autoradiography. Lanes C, PenCB, EGF, HexaCB and PMA indicate that the ^{32}P-labelled c-Jun had separated from control cells, 100 nM PenCB-treated, 10 ng/ml EGF-treated, 100 nM HexaCB-treated and 100 ng/ml PMA-treated cells, respectively. Lane M consists of ^{14}C-labelled proteins used as M_r markers (from top to bottom; BSA, 66000-M_r; ovalbumin, 46000-M_r; carbonic anhydrase, 30000-M_r).

Table 1 Stimulation of c-Jun phosphorylation after treatment with the four reagents

Increase in c-Jun phosphorylation (%) = (Radioactivity of ^{32}P-labelled c-Jun of the treated cells/Radioactivity of ^{32}P-labelled c-Jun of the control cells) × 100.

Reagent	Increase in c-Jun phosphorylation (%)	
	30 min treatment	6 h treatment
PenCB	138 ± 7.1 (n = 4)*	128 ± 18.5 (n = 3)*
EGF	171 ± 33**	142 ± 29.1 (n = 3)*
HexaCB	74 ± 12.7 (n = 3)*	100 ± 4**
PMA	76 ± 16.4 (n = 3)*	92 ± 10**

* Mean ± S.D. (n = number of experiments).
** Average of results from two independent experiments.

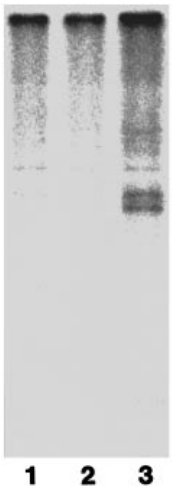

Figure 2 Specificity of immunoprecipitation

The cell lysate was prepared from cells treated with 10 ng/ml EGF for 30 min and labelled with 1 mCi/ml [^{32}P]orthophosphate. c-Jun was separated from the lysate by immunoprecipitation with normal rabbit IgG instead of anti-c-Jun IgG (lane 1), with anti-c-Jun IgG in the presence of the peptide epitope (10 μg/assay) (lane 2) and with anti-c-Jun IgG (lane 3).

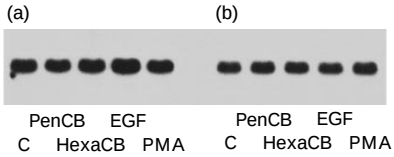

Figure 3 c-Jun mRNA levels in cells after treatment with PenCB, EGF or other reagents

The cells were treated with 100 nM PenCB (PenCB), 100 nM HexaCB (HexaCB), 10 ng/ml EGF (EGF) or 100 ng/ml PMA (PMA) for 30 min (**a**) or 6 h (**b**). Total RNA (**a**, 20 μg; **b**, 10 μg) extracted from the cells was separated on a formaldehyde/agarose gel and c-Jun mRNA levels were determined by Northern blot analysis. Lane C indicates c-Jun mRNA level in the control cells.

regulated by both treatments; however, these proteins have not been identified. On the other hand, HexaCB (100 nM, a typical non-coplanar PCB congener) did not stimulate the phosphorylation of c-Jun with the 6 h treatment (Figure 1b). Although PMA (100 ng/ml), a potent tumour promoter, is a known stimulator of AP-1 activity [29], this reagent also failed to stimulate c-Jun phosphorylation in these cells (Figure 1b). This result corresponded to our previous observation that the expression of GST-P mRNA in liver parenchymal cells was slightly elevated by treatment with PMA [18].

Even if the period of treatment with either PenCB or EGF was shortened to 30 min, identical results were obtained (Figure 1c). Both reagents rapidly stimulated the phosphorylation of c-Jun shortly after starting treatment. Neither HexaCB nor PMA increased the level of ^{32}P-labelled c-Jun after the 30 min treatment.

The radioactivities of ^{32}P-labelled c-Jun isolated from cells treated with the reagents and control cells were determined. Increases in c-Jun phosphorylation after treatment with the reagents were estimated as the ratio of the radioactivity of c-Jun of the treated cells to that of the control cells (Table 1). Phosphorylation caused by PenCB, as well as by EGF, was not greatly stimulated after either the 30 min treatment or the 6 h treatment; however, its variability among experiments was low. As expected from autoradiography, neither HexaCB nor PMA stimulated c-Jun phosphorylation after the 6 h treatment and these reagents rather reduced it after the 30 min treatment.

Phosphorylated c-Jun was not precipitated with anti-c-Jun IgG in the presence of the peptide epitope [24] (Figure 2). This band was also not detected when normal rabbit IgG was used for immunoprecipitation instead of anti-c-Jun IgG. These results indicate that c-Jun was specifically isolated from the cell lysates by immunoprecipitation.

The amount of c-Jun mRNA that had accumulated in the liver parenchymal cells was analysed after treatment with PenCB, EGF and other reagents in order to determine whether its expression was stimulated by these reagents (Figure 3). PenCB, HexaCB and PMA all failed to stimulate c-Jun mRNA expression after 30 min or 6 h treatments. However, with EGF, the amount of c-Jun mRNA increased in cells treated for 30 min, as previously reported [30]. This increased level of c-Jun mRNA was reduced to the control level after the 6 h treatment with EGF. When the cells were incubated with [^{35}S]methionine and [^{35}S]cysteine instead of with [^{32}P]orthophosphate and then treated with PenCB or EGF for 30 min, the amount of ^{35}S-labelled c-Jun in the cells did not increase with either treatment (results not shown). These results indicate that the elevation in the c-Jun-bound [^{32}P]orthophosphate by treatment with PenCB was unlikely to have been caused by an increase in the amount of c-Jun itself.

DISCUSSION

We demonstrated here that the phosphorylation of c-Jun was stimulated in primary cultured rat liver parenchymal cells by treatment with either PenCB or EGF (an effective inducer of GST-P [8,18]). However, HexaCB (a non-coplanar PCB congener), which was unable to induce GST-P, did not stimulate the phosphorylation. EGF and other growth factors activate a tyrosine kinase on their respective receptors and phosphorylate c-Jun through a signal-transducing pathway consisting of Raf, mitogen-activated protein kinase and other protein kinases, thereby elevating AP-1 activity [15–17,31]. Our results suggest that both PenCB (a coplanar PCB congener) and EGF activate protein kinases in the signal-transducing pathway and phosphorylate c-Jun, enabling it to express GST-P. Previously, Bombick et al. reported that, in mouse thymus, TCDD causes an increase in tyrosine kinase activities including c-Src activity, which is upstream of the protein kinase cascade [32]. Tyrosine-phosphorylation of cdc2 kinase, which regulates various events

of the cell cycle, was also enhanced in the liver of TCDD-administered mice [33], suggesting that TCDD may activate mitosis-related gene expression through the protein kinase cascade. However, the transcription factors phosphorylated by TCDD and related compounds have yet to be identified. c-Jun is probably the end point when this cascade is activated by the coplanar PCB congener. The phosphorylation of c-Jun may be a novel pathway for gene expression caused by this congener.

c-Jun mRNA was expressed in primary cultured liver parenchymal cells although its amount did not change with PenCB treatment, suggesting that the amount of c-Jun itself in the cells was not changed by this coplanar congener. mRNA of c-Fos, a counterpart of c-Jun in AP-1, was undetectable in these cells [19] and was not expressed even after treatment with PenCB (K. Tanno and Y. Aoki, unpublished work). Therefore, it is likely that a homodimer of phosphorylated c-Jun binds to the PRE, resulting in the expression of GST-P and other genes.

The coplanar PCB congener has a higher affinity for Ah-R than the non-coplanar form [34]. Because the phosphorylation of c-Jun was caused by the coplanar form of the congener, it may have been dependent on Ah-R. However, to date, an Ah-R-dependent pathway for protein kinase activation has not been identified. Ah-R seems to complex with c-Src and heat shock proteins 90 and 70 in the cytoplasm [35,36] and it is hypothesized that c-Src is released from the complex in an active form to stimulate protein kinases which participate in the signal-transducing pathway after TCDD binds to Ah-R [37].

Tumour promoters form a category of compounds which promote cell proliferation [29,38]. Since AP-1 regulates the expression of various genes involved in the cell cycle [29,39], phosphorylation of c-Jun by a coplanar PCB congener may cause the expression of PRE-containing genes which act to disregulate the cell cycle, resulting in tumour-promoting activity and various other adverse effects. Sutter et al. cloned plasminogen activator inhibitor 2 and interleukin 1β as the genes expressed as a result of treating keratinocytes with TCDD [40]. These genes were also inducible by treatment with PMA [41,42], suggesting that some features common to TCDD and tumour promoter activities are found in the regulation of expression of these genes. In order to understand the various toxic effects of the coplanar PCB congener and related compounds, identification of the protein kinase activated by this congener and the target gene expressed through the protein kinase cascade are required.

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