Epidermal growth factor regulation of glutathione S-transferase gene expression in the rat is mediated by class Pi glutathione S-transferase enhancer I

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Using chloramphenicol acetyltransferase assays we showed that epidermal growth factor (EGF), transforming growth factor α (TGFα), and 3,3′,4,4′,5-pentachlorobiphenyl (PenCB) induce class Pi glutathione S-transferase (GSTP1) in primary cultured rat liver parenchymal cells. GSTP1 enhancer I (GPEI), which is required for the stimulation of GSTP1 expression by PenCB, also mediates EGF and TGFα stimulation of GSTP1 gene expression. However, hepatocyte growth factor and insulin did not stimulate GPEI-mediated gene expression. On the other hand, the antioxidant reagents butylhydroxyanisole and *t*-butylhydroquinone, stimulated GPEI-mediated gene expression, but the level of GSTP1 mRNA was not elevated. Our observations suggest that EGF and TGFα induce GSTP1 by the same signal transduction pathway as PenCB. Since the sequence of GPEI is similar to that of the antioxidant responsive element (ARE), some factors which bind to ARE might play a role in GPEI-mediated gene expression.

Key words: antioxidant responsive element, coplanar polychlorinated biphenyl, chloramphenicol acetyltransferase assay, primary culture, transfection.

INTRODUCTION

Glutathione S-transferase is one of the Phase II detoxifying enzymes involved in the defense mechanism against xenobiotics [1,2]. Class Pi glutathione S-transferase (GSTP1), which is latent in normal rat liver, is a marker enzyme for pre-neoplastic hepatic foci and hepatocarcinomas [3,4]. Previously, we showed that GSTP1 was specifically induced in primary cultured rat liver parenchymal cells by 3,3′,4,4′,5-pentachlorobiphenyl (PenCB) [5], one of the most toxic coplanar polychlorinated biphenyl congeners [6,7].

Coplanar polychlorinated biphenyl congeners and related compounds (e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) are dioxin-related compounds (dioxins), which exhibit various kinds of toxicity, such as hepatocarcinogenicity [6–8] and teratogenicity [9,10]. Recently, these compounds were recognized as disruptors of the endocrine system [11,12]. We have been interested in clarifying the signal transduction pathways which may play roles in the common mechanisms involved in the induction of various toxicities by dioxins. A well known mechanism for gene expression induced by dioxins involves an aryl hydrocarbon receptor (AhR)-mediated pathway [13,14]. Dioxins taken up by cells bind to AhR (a member of the per AhR nuclear translocator Sim homology region family of ligand-dependent transactivating factors), and the ligand–receptor complex translocates into the nucleus and associates with AhR nuclear translocator factor (another member of the per AhR nuclear translocator Sim homology region family). The resulting heterodimer acts on a specific region, termed the xenobiotic-responsive element, in the 5′-flanking sequences of cytochrome P450 1A1, glutathione S-transferase A2 subunit and some other Phase II enzyme genes, causing their expression [15]. However, since the 5′-flanking sequence of the *GSTP1* gene does not contain an xenobiotic-responsive element [16,17], AhR may not act directly as a transactivating factor for *GSTP1* gene expression. As PenCB is difficult to metabolize [18], its metabolite probably does not stimulate gene expression. It seems that PenCB itself stimulates some gene expression, and this gene product stimulates *GSTP1* gene expression. We expected to find a novel signal transduction pathway activated by dioxins, and by identifying its components, to reveal the mechanism for the induction of GSTP1.

Previously we have shown PenCB- and epidermal growth factor (EGF)-induced expression of GSTP1 mRNA in primary cultured rat liver parenchymal cells [19]. It was expected that PenCB and EGF induce GSTP1 by a common mechanism. Using a chloramphenicol acetyltransferase (CAT) assay we identified an enhancer element, termed GSTP1 enhancer I (GPEI), in a 5′-upstream region which is required for GSTP1 induction in rat liver parenchymal cells by PenCB [20]. The region is located 2.5 kb upstream of the transcription initiation site of the *GSTP1* gene, and was originally identified as the element necessary for GSTP1 expression in hepatocarcinoma cells [21,22]. GPEI contains a dyad of PMA-responsive element (TRE)-like elements palindromically oriented with a 3 bp interval [21,23]. This TRE-like sequence has recently been recognized to be similar to the antioxidant responsive element (ARE) consensus or nuclear factor erythroid 2 consensus which is required for gene expression in response to antioxidant reagents such as butylhydroxyanisole (BHA) and *t*-butylhydroquinone (t-BHQ) [24–26]. We determined whether GPEI is required for GSTP1

Abbreviations used: AhR, aryl hydrocarbon receptor; ARE, antioxidant responsive element; BHA, butylhydroxyanisole; t-BHQ, *t*-butylhydroquinone; CAT, chloramphenicol acetyltransferase; EGF, epidermal growth factor; β-Gal, *Escherichia coli* β-galactosidase; GPEI, class Pi glutathione S-transferase enhancer I; GPEIs, 38 bp core sequence of GPEI; GSTP1, rat class Pi glutathione S-transferase; HGF, hepatocyte growth factor; MAP, mitogen-activated protein; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; PenCB, 3,3′,4,4′,5-pentachlorobiphenyl; TGFα, transforming growth factor-α; TRE, PMA-responsive element; XRE; xenobiotic-responsive element.

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induction by EGF, other growth factors and antioxidant reagents, and report here that EGF and transforming growth factor α (TGFα) stimulate GPEI-mediated gene expression.

MATERIALS AND METHODS

Chemicals

GPEI/Δ–56kCAT plasmid, its synthetic fragment and mutants, and pGP5 were prepared as described previously in [21,23]. Vector pSVβGAL and TransFast™ transfection reagent were purchased from Promega; mouse EGF and acetyl coenzyme A were from Wako Pure Chemicals (Tokyo, Japan); recombinant human hepatocyte growth factor (HGF) was from Becton Dickinson (Bedford, MA, U.S.A.); rat TGFα was from Calbiochem; insulin, BHA, dexamethasone, and cell culture media were from Sigma; t-BHQ was from Aldrich (Milwaukee, WI, U.S.A.); fetal bovine serum was from Life Technologies; 1-deoxy[dichloroacetyl-1-^{14}C]chloramphenicol was from Amersham International; and β-galactosidase reporter gene assay kit was from Boehringer Mannheim.

Preparation of monolayer cultures of rat liver parenchymal cells

Cell culture was performed essentially as described in [19]. Liver parenchymal cells were isolated from the liver of male Wistar rats by digestion with collagenase. Cells (2.5×10^6) in 3 ml of modified Williams medium E (pH 7.4), containing 10 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 25 mM Hepes/NaOH buffer, 1 nM insulin, 100 nM dexamethasone, and 10% (v/v) fetal bovine serum, were plated in collagen-coated 60 mm plates. After the cells had been cultured for 4 h at 37 °C in air/CO_2 (19:1, v/v), the medium was changed to serum-free Williams medium E, containing 1 nM insulin, 100 nM dexamethasone, and 0.1 μg/ml aprotinin, and the cells were then incubated for 20 h.

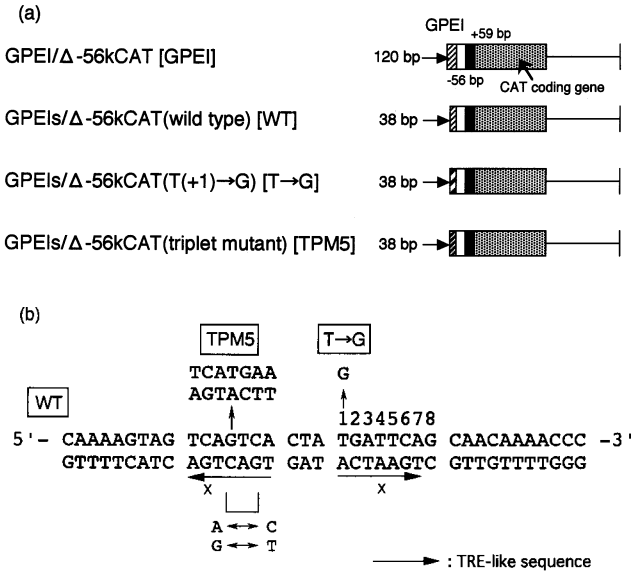

Figure 1 Structures of GPEI and GPEIs–CAT fusion plasmid constructs

(**a**) Structures of the constructs used for transfections. The abbreviations in the square brackets show the plasmid DNA species used for the results shown in Figure 4. (**b**) Structures of GPEIs and its synthetic mutants [21]. The TRE-like sequence is indicated by horizontal arrows. The positions of nucleotides in the TRE-like sequence are numbered in accordance with Angel et al. [48]. The single point-mutated nucleotide is indicated by a vertical arrow. TPM5 is the mutant in which the nucleotides as a consecutive triplet are changed to non-complementary ones (A↔C, G↔T). The TPM5 sequence is shown above the wild-type sequence. The positions of nucleotides different from those of the consensus TRE are indicated by X.

Construction of reporter gene plasmids

CAT fusion plasmids were constructed by the method of Sakai et al. [23]. GPEI fragments, a synthetic 38 bp fragment of the GPEI core, and its mutants were linked to Δ-56kCAT (an enhancer-negative plasmid that contains a GC box and a TATA box) by the method of Okuda et al. [21]. The structures of GPEI/Δ–56kCAT, GPEIs/Δ–56kCAT and its mutants are illustrated in Figure 1. pSVβGAL includes the SV40 promoter and enhancer [27] and *lac*Z [*Escherichia coli* β-galactosidase (β-Gal)] as a reporter gene.

DNA transfection and treatment with growth factors and antioxidant reagents

Plasmid transfections of monolayer cultures of rat liver parenchymal cells were performed by the lipofection method [28–30]. Cells were washed with 3 ml of Williams medium E without fetal bovine serum before lipofection. The plasmids were then transfected into the cells with 1 ml of Williams medium E without fetal bovine serum and 24 μl of TransFast reagent per 60 mm plate. CAT plasmids (6 μg) were transfected into the cells with 2 μg of pSVβGAL. After the cells were incubated for 1 h at 37 °C in air/CO_2 (19:1), 2 ml of serum-free Williams medium E, containing 10 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 25 mM Hepes/NaOH buffer, 1 nM insulin, 0.1 μg/ml aprotinin, and 6 mM nicotinamide was added. Cells were treated with growth factors, BHA, t-BHQ or PenCB (which were added to the medium at the indicated concentrations as described in [19]), and incubated for an additional 36 h.

Cells were harvested by scraping in PBS. Cell extracts were prepared by four cycles of freeze–thawing in 0.25 M Tris/HCl (pH 7.5), and their protein concentrations were determined by the procedure of Bradford [31].

Reporter gene assay

For CAT assays [32], the extracts were heated at 65 °C for 10 min, and the precipitates were removed by centrifugation at 15000 ***g*** for 5 min. The reaction mixtures (final volume 125 μl), containing cell extract, 210 mM Tris/HCl (pH 7.5), 11 kBq of 1-deoxy[^{14}C]chloramphenicol [33] and 80 nmol of acetyl-CoA, were incubated at 37 °C for 2 h. Reactions were terminated by the addition of 1 ml of cold ethyl acetate. The product (3-acetyl-1-deoxychloramphenicol) and unreacted substrate were extracted with ethyl acetate. After evaporation of the ethyl acetate, the residue was dissolved in 20 μl of ethyl acetate, and chromatographed on a thin layer plate (LK6D; Whatman, Maidstone, Kent, U.K.) with chloroform/methanol (94:6, v/v). The radioactivity of product and substrate was analysed using a Fuji BAS 2000 system (Fuji Photo, Tokyo, Japan). CAT activity was expressed as the ratio of radioactivity of the product to the total radioactivity. We usually use β-Gal activity to standardize CAT activity; however, the expression of plasmids that we used as controls previously [20] was stimulated in cultured liver parenchymal cells by treatment with EGF. Therefore CAT activity was standardized with protein concentration of the cell extract.

β-Gal activity in the cell extracts was measured as described previously [20].

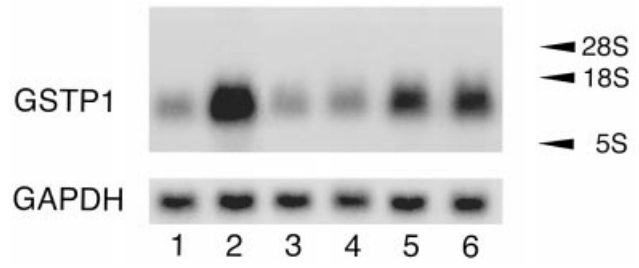

Figure 2 Induction of GSTP1 mRNA by PenCB, antioxidant reagents and growth factors

Rat liver parenchymal cells were treated with PenCB (100 nM), BHA (60 μM), t-BHQ (60 μM), EGF (10 ng/ml), or TGFα (10 ng/ml) for 36 h after being cultured for 25 h. GSTP1 and glyceraldehyde-3-phosphate dehydrogenase mRNA levels in the cells were determined by Northern-blot analysis. The lanes were loaded with 20 μg of RNA from the cells treated as follows: 1, control; 2, PenCB (100 nM); 3, BHA (60 μM); 4, t-BHQ (60 μM); 5, EGF (10 ng/ml); 6, TGFα (10 ng/ml). The positions of 28 S, 18 S, and 5 S ribosomal RNA are indicated at the right.

RNA preparation and Northern-blot analysis

When the level of GSTP1 mRNA induced in primary cultured rat liver parenchymal cells by a growth factor, BHA, t-BHQ, or PenCB was determined, untransfected cells were cultured in the presence of the inducing agent in the same manner as transfected cells. After the cells had been treated with the agent for 36 h, total RNA was extracted from the cells as described by Chomczynski and Sacchi [34]. A 20 μg portion of RNA was separated on a formaldehyde/1.2% agarose gel containing 0.02% ethidium bromide. After RNA was separated on the gel, the positions of ribosomal RNAs were visualized by UV illumination. The separated RNA was transferred to a nylon membrane (Amersham), and the membrane was incubated with a GSTP1 cDNA probe, which is an *Eco*RI–*Sal*I fragment of pGP5 [35], as described in [19,36]. The membrane was rehybridized with glyceraldehyde-3-phosphate dehydrogenase probe [19]. Radioactive bands on the membrane were detected by autoradiography.

RESULTS

Primary cultured rat liver parenchymal cells were treated with PenCB (100 nM), BHA (60 μM), t-BHQ (60 μM), EGF (10 ng/ml), or TGFα (10 ng/ml; a ligand of the EGF receptor) for 36 h, and the relative amounts of GSTP1 mRNA were determined by Northern blotting (Figure 2). Expression of GSTP1 mRNA was stimulated in the cells treated with EGF or TGFα, as well as with PenCB. However, it was not stimulated in the cells treated with BHA or t-BHQ.

We found previously that the expression of a CAT plasmid containing the -2.9 kb upstream region of the GSTP1 gene was stimulated in rat liver parenchymal cells by EGF as well as by PenCB [20]. Since this stimulation of CAT gene expression by PenCB was dependent on the GPEI element, we determined whether expression of the GPEI/Δ–56kCAT plasmid (120 bp region around GPEI core-ligated to Δ–56kCAT plasmid) was stimulated by EGF, as was shown with PenCB. As expected, the expression of GPEI/Δ–56kCAT was increased 1.73-fold in cells treated with EGF (Figure 3a), and the extent of the increase caused by EGF was similar to that caused by PenCB (1.52-fold) (results not shown). This is consistent with the similarity in the levels of GSTP1 mRNA after treatment with EGF or PenCB. EGF probably stimulates GPEI-mediated gene expression, suggesting that GPEI is required for the increase in GSTP1 expression caused by EGF.

To examine this possibility, CAT activities were determined in cells transfected with GPEIs/Δ–56kCAT plasmid [Figure 1, GPEI core (38 bp) conjugated to Δ-56kCAT plasmid] after treatment with EGF or TGFα (Figure 3b). CAT expression was

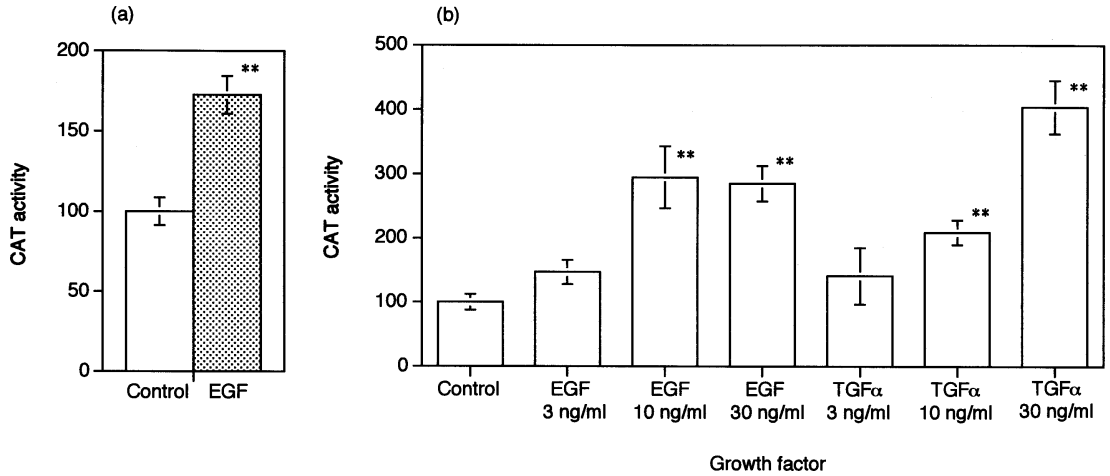

Figure 3 Effect of growth factors on the expression of *CAT* in primary cultured rat liver parenchymal cells transfected with GPEI or GPEIs (WT)–CAT construct

(**a**) Effect of EGF on the cells transfected with GPEI–CAT construct. Rat liver parenchymal cells were transfected with 6 μg of GPEI–CAT construct on each 60 mm plate, then treated with 10 ng/ml of EGF for 36 h. CAT activity was assayed and standardized with protein concentration in the cell extract. Each value is the mean $\pm$ S.E.M. for five separate experiments. Open bars show the CAT activity of the cells without EGF treatment (control); stippled bars show that of the cells treated with EGF. The activity in the cells without EGF treatment (control) is represented as 100. (**b**) Effect of the concentration of EGF and TGFα on the cells transfected with GPEIs–CAT construct. Rat liver parenchymal cells were transfected with 6 μg of GPEIs–CAT construct on each 60 mm plate, then treated with EGF or TGFα for 36 h. CAT activity was assayed and standardized as above. Each value is the mean $\pm$ S.E.M. for five separate experiments. The activity in the cells without a growth factor treatment (control) is represented as 100. $^{**}P < 0.01$ compared with control.

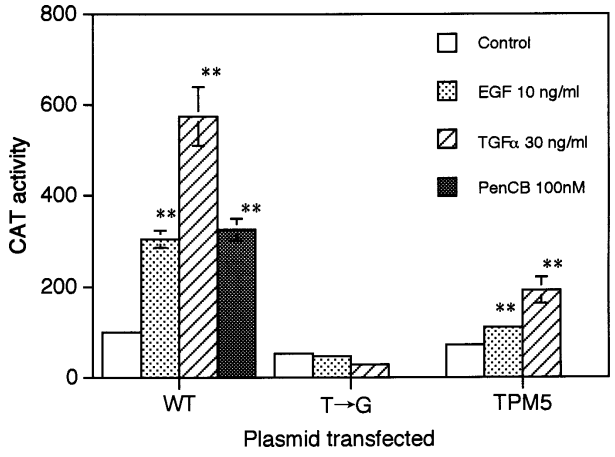

Figure 4 Effect of EGF and TGFα on the expression of *CAT* in primary cultured rat liver parenchymal cells transfected with wild-type GPEIs or mutant GPEIs–CAT constructs

Rat liver parenchymal cells were transfected with 6 μg of CAT construct on each 60 mm plate, then treated with PenCB, EGF, or TGFα for 36 h. The structures and abbreviated names of the plasmid constructs are shown in Figure 1. Each value is the mean ± S.E.M. for five separate experiments. The activity in the cells transfected with wild-type GPEIs plasmid (WT) without growth factor treatment is represented as 100. $^{**}P < 0.01$ compared with control.

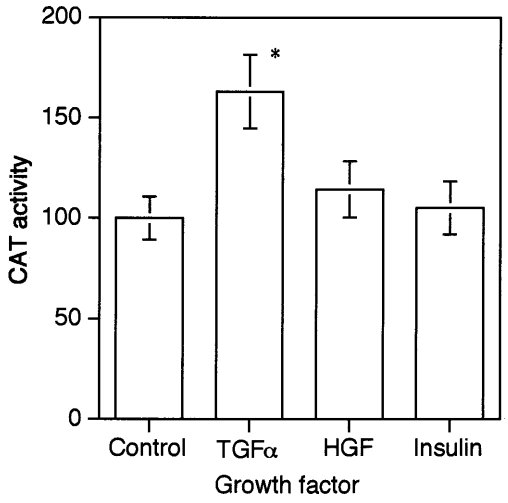

Figure 5 Effect of growth factors on the expression of *CAT* in primary cultured rat liver parenchymal cells transfected with wild-type GPEIs–CAT construct

Rat liver parenchymal cells were transfected with 6 μg of wild-type GPEIs–CAT construct on each 60 mm plate, then treated with a growth factor for 36 h. Each value is the mean ± S.E.M. for five separate experiments. The activity in the cells without growth factor treatment (control) is represented as 100. $^{*}P < 0.05$ compared with control.

stimulated in cells treated with EGF at concentrations of 10 and 30 ng/ml; TGFα also stimulated CAT expression in a dose-dependent manner at the same concentrations. At 30 ng/ml, TGFα stimulated CAT expression approx. 4-fold. In cells treated with 10 ng/ml EGF, CAT activity was the same as in cells treated with 100 nM PenCB (Figure 4, WT). The optimum concentration of EGF or TGFα for stimulating DNA synthesis in cultured liver parenchymal cells is 10–30 ng/ml, but 100 nM PenCB was not effective in this regard (results not shown).

These results show that the induction of GSTP1 in rat liver cells by EGF and TGFα is mediated by the GPEI enhancer element core. To confirm this, CAT assays were performed after treatment of cells with EGF (10 ng/ml) or TGFα (30 ng/ml), which had been transfected with the mutant plasmids of GPEIs/Δ–56kCAT (termed T → G and TPM5), which have a single point mutation in the downstream TRE-like sequence of GPEI and three consecutive point mutations in the upstream TRE-like sequence respectively (Figure 1). As shown in Figure 4, while CAT expression was stimulated by both EGF and TGFα in the cells transfected with the GPEIs/Δ–56kCAT (wild-type) plasmid, in the cells transfected with the T → G mutant plasmid CAT expression was not stimulated by either growth factor. However, in the cells transfected with the TPM5 mutant plasmid CAT expression was slightly stimulated.

CAT activities were also determined in rat liver parenchymal cells transfected with GPEIs/Δ–56kCAT (wild-type) plasmid and treated with HGF (20 ng/ml), which stimulates DNA synthesis in liver parenchymal cells [37], or with insulin (100 nM). CAT expression was stimulated in the cells transfected with GPEIs/Δ–56kCAT after treatment with TGFα, but not in those treated with HGF or insulin (Figure 5), indicating that GPEI-mediated gene expression is caused specifically by EGF and related ligands.

To examine the responsiveness of GPEI to antioxidant reagents, CAT activities were determined in rat liver cells transfected with GPEIs/Δ–56kCAT (wild-type) plasmid or its mutant plasmids (T → G, TPM5) and treated with BHA (60 μM) or t-BHQ (60 μM). At 60 μM of t-BHQ, GPEI-mediated gene expression was stimulated in HepG2 cells [26]. As shown in

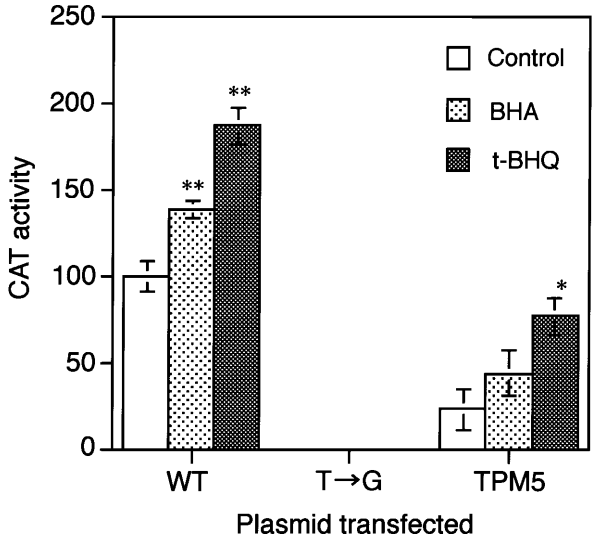

Figure 6 Effect of antioxidant reagents on the expression of *CAT* in primary cultured rat liver parenchymal cells transfected with wild-type GPEIs or mutant GPEIs–CAT constructs

Rat liver parenchymal cells were transfected with 6 μg of CAT construct on each 60 mm plate, then treated with BHA or t-BHQ for 36 h. The structures and abbreviated names of the plasmid constructs are shown in Figure 1. Each value is the mean ± S.E.M. for four separate experiments. Open bars show the CAT activity of the cells without antioxidant treatment (control); stippled bars of the cells treated with BHA (60 μM); shaded bars of the cells treated with t-BHQ (60 μM). The activity in the cells transfected with wild-type GPEIs plasmid (WT) without antioxidant treatment is represented as 100. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control.

Figure 6, CAT expression was also stimulated by both BHA and t-BHQ in rat liver cells transfected with GPEIs/Δ–56kCAT (wild-type) plasmid. The expression was stimulated more by t-BHQ than by BHA. In the cells transfected with the T → G mutant plasmid CAT expression was not stimulated by either antioxidant, but in the cells transfected with the TPM5 mutant plasmid CAT expression was slightly stimulated by both antioxidant reagents. This was similar to the results with EGF and TGFα.

DISCUSSION

We demonstrated that EGF and TGFα stimulate GPEI-mediated gene expression in primary cultured rat liver parenchymal cells, indicating that GPEI, an ARE-like enhancer element, is required for the stimulation of GSTP1 expression by EGF or TGFα. The GPEI enhancer element is located 2.5 kb upstream of the initiation site of the GSTP1 gene. Both EGF and TGFα (a ligand of the EGF receptor) activate the tyrosine kinase of the EGF receptor for potentiating signal transduction pathways, e.g. the MAP (mitogen-activated protein) kinase cascade [38]. GPEI-mediated gene expression might be stimulated by EGF through these signal transduction pathways.

We showed previously that the GPEI element is required for PenCB-induced expression of the *GSTP1* gene in liver parenchymal cells [20], as it is for GSTP1 expression in hepatocarcinoma. Since GPEI-mediated gene expression was stimulated by both EGF and PenCB, it is possible that PenCB mimics the effect of EGF by activating the same signal transduction pathway as EGF, resulting in the stimulation of the expression of the *GSTP1* gene. It is well known that EGF activates c-Jun, a transcription factor which binds to TRE as a heterodimer with c-Fos, through the phosphorylation of c-Jun by MAP kinases [39]. Previously, we showed that in cultured liver parenchymal cells phosphorylation of c-Jun was stimulated by PenCB or EGF treatment [40]. However, we have not clarified whether this stimulation by PenCB depends on stress-activated protein kinase, c-Jun N-terminal kinase, or other protein kinases. Activation of the ARE-protein complex by Phase II enzyme inducers is also regulated by signal transducing kinase cascades [2]. It has been reported that the extracellular signal-regulated protein kinase 2 kinase pathway is involved in the ARE-mediated induction of Phase II detoxifying enzymes by t-BHQ [41]. The signal transduction pathway for phosphorylating c-Jun through MAP kinases and related kinases seems to be the common pathway for stimulating GSTP1 expression by PenCB and EGF. c-Jun has been shown to regulate the expression of genes containing an ARE in co-ordination with transcription factors such as nuclear factor erythroid 2-related factor 2 (Nrf2) and c-Fos [42]. In particular, Itoh et al. reported that Nrf2 is essential for the expression of some Phase II drug-metabolizing enzymes containing an ARE [25]. Venugopal and Jaiswal [43] also showed that Nrf2 acts on the ARE of the human *NAD(P)H:quinone oxidoreductase* 1 (*NQO1*) gene along with some factors. The human and rat NQO1 promoters have two AREs which are classed as 'ARE enhancers found in inverted-repeat orientation' [44]. The sequence of the GPEI is similar to those of the NQO1 promoters. However, there are two nucleotide differences between the prototype ARE (5′-GTGACNNNGC-3′) [45] and the GPEI (5′-ATGATTCAGC-3′). The transcription factors such as c-Jun and Nrf2 may also contribute to GPEI-mediated gene expression by EGF, but it is not understood how *GSTP1* gene expression mediated by the GPEI element is regulated by EGF and PenCB, although the transcription factors involved in the expression of ARE-containing genes (e.g. NQO1) stimulated by xenobiotics have been examined extensively.

We have shown that the influence of EGF or TGFα on the expression of the CAT gene in the cells transfected with the mutant GPEIs/Δ–56kCAT plasmids was different from that of PenCB. While PenCB did not stimulate CAT activity in cells transfected with either of the mutant GPEIs plasmids, T → G and TPM5, EGF and TGFα did not stimulate CAT activity in the cells transfected with the T → G mutant plasmid, but did stimulate the activity a little in cells transfected with the TPM5 mutant plasmid. Since the single point mutation (T → G) is located in the downstream TRE-like sequence of GPEI, EGF seems to stimulate the expression of GSTP1 via the downstream TRE-like sequence, while both TRE-like sequences are required for the stimulation of GSTP1 expression by PenCB. In GPEI, the downstream TRE-like sequence is very close to a perfect TRE, but the upstream half-site is an imperfect TRE [22,44]. Both TRE-like sequences are required for full activity of GPEI; the downstream sequence is essential for GSTP1 expression. Therefore the T → G mutant plasmid is probably not responsive. On the other hand, the TPM5 mutant plasmid may be responsive to c-Jun because the downstream TRE-like sequence becomes a typical TRE. c-Jun might mediate the slightly stimulated CAT expression in the cells transfected with the TPM5 mutant plasmid.

Since HGF or insulin did not stimulate GPEI-mediated CAT expression, EGF appears to be a growth factor which specifically effects the expression of genes containing a GPEI. We showed that EGF has the potential to activate the expression of the gene containing an ARE-like element as well as a TRE in its 5′-flanking sequence. Crosstalk between signal transduction pathways activated by EGF and an ARE might occur in liver parenchymal cells.

BHA and t-BHQ stimulated GPEI-mediated CAT expression. The effect of BHA or t-BHQ on CAT expression in the cells transfected with the GPEIs/Δ–56kCAT (wild-type) plasmid and its mutant plasmids was similar to that of EGF or TGFα. GPEI seems to be responsive to BHA or t-BHQ as well as to EGF. However, the level of GSTP1 mRNA was not elevated in the cells treated with BHA or t-BHQ. An additional pathway other than GPEI-mediated regulation may be required for elevation of GSTP1 mRNA levels by PenCB and EGF; however, BHA and t-BHQ seem to be unable to activate this additional pathway. For example, a silencer element is located in the 5′-flanking region of the *GSTP1* gene [21], and PenCB and EGF possibly abolish the effect of the silencer. Sherratt et al. showed that GSTP1 was induced in the livers of rats fed a diet containing 0.75 % BHA for 14 days [46]. The expression of GSTP1 in rat liver could have been caused by a longer exposure to BHA than is the case in primary cultured liver cells.

As mentioned above, Nrf2 is thought to interact with c-Jun or the transcriptional factor, Maf [25], for the expression of ARE-containing genes. Recently, Nrf2 was shown to be located in the cytoplasm, associated with Keap1 factor which possibly binds to actin. This complex is translocated into the nucleus in response to the pro-oxidant, diethylmaleate, resulting in the expression of ARE-containing Phase II drug-metabolizing enzymes [47]. We have already shown that a signal transduction pathway including a protein kinase cascade is activated by PenCB as well as EGF for phosphorylating c-Jun [40]. Although the contribution of Nrf2 to the expression of the *GSTP1* gene is unclear, a signal transduction pathway activated by EGF or PenCB could activate the Nrf2/Keap1 system to induce the expression of GSTP1 and related ARE-containing genes. Further studies are required to elucidate the mechanism by which the expression of GPEI or ARE-containing genes is regulated by EGF and PenCB.

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