GPE (GSTP enhancer), located approx. 2.5 kb upstream from the cap site, and the silencer [30,31]. GPE1, a strong enhancer element in GPE, is responsible for GSTP gene expression during hepatocarcinogenesis *in vivo* [32,33]. Recently, we showed that a heterodimer comprising Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) and MafK binds to GPE1 and enhances GSTP promoter activity [34]. Nrf2, a member of bZIP (basic region leucine zipper) family of transcription factors, induces Phase II detoxifying and antioxidative genes [35]. Nrf2 plays a crucial role in early defence against chemical stress and carcinogenesis [36].

To characterize the roles of HATs during hepatocarcinogenesis, we examined their expression profiles and showed that expression of MOZ was induced under these conditions. Further, we found that MOZ acted as a co-activator of the Nrf2–MafK heterodimer and induced expression of GSTP. These results suggest that MOZ induces GSTP expression through the Nrf2-mediated pathway during early hepatocarcinogenesis.

EXPERIMENTAL

Chemical hepatocarcinogenesis of rats

Carcinogenic experiments were performed according to the Solt–Farber protocol [37]. Experiments were initiated by intraperitoneal injection of DEN (diethylnitrosoamine; 200 mg/kg) into 5-week-old Wister rats. After the animals had been fed basal diets for 2 weeks, diets were changed to basal diets containing 0.02 % AAF (2-acetylaminofluorene). Three weeks after the DEN injection, a PH (partial hepatectomy) was performed; livers were extirpated 7 weeks after the DEN injection. Control rats were injected with saline and fed basal diets. All animal care and handling procedures were approved by the Animal Care and Use Committee of Osaka University.

Preparation of nuclear extracts, cytosol fractions and RNA from rat liver

Procedures for preparation of nuclear extracts and cytosol fractions from rat liver were described previously [38]. Livers were homogenized in a sucrose-containing buffer, and nuclei were purified by centrifugation. Nuclear proteins were extracted with 0.55 M KCl and centrifuged at 40000 g for 60 min at 4 °C. The supernatants were used for the HAT assay and Western blot analysis. Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.A.) in accordance with the manufacturer's recommendations.

Western blotting and antibodies

Proteins were resolved using SDS/PAGE, transferred to nitrocellulose or PVDF membrane and detected using the ECL® (enhanced chemiluminescence) Western blotting analysis detection system (Amersham Biosciences, Piscataway, NJ, U.S.A.). For the generation of antibodies against the N- and C-terminal regions of MOZ, nucleotides corresponding to amino acid residues 1–331 and 1717–1998 respectively were cloned into pET-28a (Novagen, Darmstadt, Germany). The resulting His_6-tagged fusion polypeptides were expressed in bacteria and purified over nickel-nitrilotriacetic acid–agarose (Qiagen, Hilden, Germany). These proteins were injected into rabbits, and antibodies were affinity-purified using Protein A–Sepharose (Amersham Biosciences). The anti-P/CAF antibody was a gift from Dr Y. Nakatani (Harvard Medical School, Boston, MA, U.S.A.). The following antibodies were commercially available: anti-p300 (N-15, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-CBP (A-22, Santa Cruz Biotechnology), anti-GCN5 (N-18, Santa Cruz Biotechnology), anti-TIP60 (Upstate Biotechnology, Lake Placid, NY, U.S.A.), anti-MORF (MOZ-related factor; C-15, Santa Cruz Biotechnology), anti-MYST (Upstate Biotechnology), anti-GSTP (Biotrin, Dublin, Ireland), anti-HA (haemagglutinin) (6B12, Babco, Berkeley, CA, U.S.A.), and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (MAB374, Chemicon, Temecula, CA, U.S.A.).

Plasmid construction

The rat MOZ expression plasmid pCI-MOZ has been described previously [39]. Mutants within the PHD (plant homeodomain) finger and the MYST regions of the *Moz* gene (pCI-MOZ-PHDmut and pCI-MOZ-MYSTmut) were generated using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) following the manufacturer's recommended protocols. All mutations were verified by sequencing over the region of change. For construction of Myc-tagged MOZ-expressing plasmids, the MOZ ORF (open reading frame) was subcloned into the EcoRI–NotI site of pCMV-Myc (Clontech, Franklin Lakes, NJ, U.S.A.). For construction of −2.5GST-luciferase, the fragment from −2.5 kb to −91 kb of the GSTP gene [30] was inserted into the SacI site of −91GST-luciferase [38]. To generate −2.15GST-luciferase (the GPE deletion reporter plasmid), −2.5GST-luciferase was digested with SmaI and AccI, blunted with Klenow fragment (Toyobo, Osaka, Japan), and then self-ligated. The Nrf2 expression plasmid (pAβ2-Nrf2), including the human β-actin promoter and enhancer, and GPE1 reporter plasmid (GPE1-luciferase) were described previously in [40]. The HA-tagged rat MafK expression plasmid (pCMV-HA-MafK) was generated by PCR amplification of the MafK ORF [40] using primers that incorporate SalI and NotI at the 5′ and 3′ ends respectively. The PCR product was cloned into the SalI–NotI site of pCMV-HA (Clontech). The non-tagged MafK expression plasmid pRSV-MafK contained MafK cDNA controlled by the *Rous sarcoma* virus long terminal repeat. For construction of MafK/GEX-KG, MafK cDNA was cloned into pGEX-2T (Amersham Biosciences).

Cell culture

Rat hepatoma H4IIE cells and mouse embryonic carcinoma F9 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FBS (fetal bovine serum). HeLa cells were cultured in minimal essential medium supplemented with 10 % FBS.

Transfection and reporter gene assays

Transfection of H4IIE and F9 cells was performed using FuGENE™ 6 (Roche, Indianapolis, IN, U.S.A.) in accordance with the manufacturer's instructions. For H4IIE cells, all transfections included 100 ng of the reporter plasmid, with or without 1 μg of the MOZ expression plasmid (pCI-MOZ). The amount of plasmid in the transfection was kept constant by using empty pCI vector. Transfectants were harvested 48 h after transfection. The luciferase assay was performed as described previously [38] and protein concentrations were determined by the method of Bradford. Luciferase activities were normalized to the protein amount. In some experiments, the transfection efficiency was checked by co-transfection with pRSV-GAL, a eukaryotic expression plasmid that contained the *Escherichia coli* β-galactosidase structural gene controlled by the *Rous sarcoma* virus long terminal repeat. β-Galactosidase activity was assayed as described in [38]. We confirmed that the variation of transfection efficiency was <15 %. Relative luciferase activity was estimated by the luciferase activity from −2.5GST-luciferase in the absence of MOZ.

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For F9 cells, all transfections contained 100 ng of reporter plasmid (GPE1-luciferase) and 5 ng of *Renilla* luciferase plasmid phRL-tk (Promega, Madison, WI, U.S.A.) as an internal control to normalize for transfection efficiency, with or without 1 μg of the MOZ expression plasmid (pCI-MOZ) in the presence or absence of 5 ng of the Nrf2 expression plasmid (pAβ2-Nrf2). The amount of plasmid in the transfection was kept constant by using empty pCI vector. At 48 h after transfection, cells were harvested and assayed for luciferase activity using the Dual-luciferase Reporter Assay System (Promega) in accordance with the manufacturer's recommendations. Reported values are relative to the activity of GPE1-luciferase without transfection of Nrf2 and MOZ. All experiments were repeated at least three times with two or three different preparations of DNA.

GST pull-down assay

The recombinant plasmid was transformed into BL21(DE3)pLysS cells. Transformants were grown overnight at 30 °C in Luria–Bertani medium containing 100 μg/ml ampicillin. The culture then was diluted 25-fold and grown to an attenuance (D_{600}) of 0.4; at that time, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. The cells were allowed to grow for an additional 4.5 h and then were harvested by centrifugation; resuspended in a buffer containing 0.15 M KCl, 50 mM Tris (pH 8.0), 10 % (v/v) glycerol, 0.1 % Tween 20, 1 mM DTT (dithiothreitol) and 1 mM PMSF, and disrupted by sonication. After centrifugation at 7000 ***g*** for 10 min, the supernatant was cross-linked to glutathione–Sepharose 4B with dimethylpimelimidate. ^{35}S-labelled MOZ proteins were produced using pCI-MOZ, pCI-MOZ-PHDmut and pCI-MOZ-MYSTmut as templates by *in vitro* transcription–translation with the TNT T7-coupled reticulocyte lysate system (Promega). A 5 μl aliquot of the reticulocyte lysate reaction containing ^{35}S-labelled MOZ proteins was incubated for 3 h at 4 °C in a buffer containing 0.15 M KCl, 50 mM Tris (pH 7.6), 10 % (v/v) glycerol, 1 mM DTT and 1 mM PMSF with GST fusion proteins. After extensive washes, bound proteins were separated by SDS/PAGE and detected by autoradiography.

Immunoprecipitation assay

Myc-tagged MOZ expression plasmid (pCMV-Myc-MOZ) was co-transfected into HeLa cells with HA-tagged MafK (pCMV-HA-MafK) or non-tagged MafK (pRSV-MafK) by the calcium phosphate co-precipitation method [41]. The cells were harvested 48 h after transfection, and nuclear extracts from the transfected HeLa cells were prepared as described in [38]. Nuclear extracts were diluted by adding nuclear lysis buffer containing 20 mM Hepes (pH 7.9), 1 mM EDTA, 0.5 mM spermidine, 1 mM DTT, 10 % glycerol, 1 mM PMSF, 1 μg/ml pepstatin A and 1 μg/ml leupeptin (final KCl concentration, 0.15 M). To immunoprecipitate HA-tagged protein, we incubated extracts with anti-HA antibody immobilized on Sepharose beads overnight at 4 °C. For control experiments, control mouse IgG coupled with Sepharose was used. After extensive washes, bound proteins were separated by SDS/PAGE and detected by Western blotting.

Induction of endogenous GSTP expression by MOZ in rat hepatoma H4IIE cells

Rat hepatoma H4IIE cells were transfected with various amounts of the MOZ expression plasmid pCI-MOZ by using the FuGENE^{TM} 6 reagent in 35 mm plates. The total amount of plasmid DNA was adjusted by supplementing with empty pCI vector to 1 μg. After 36 h, cell lysates were prepared with a buffer comprising 25 mM Tris phosphate (pH 7.8), 2 mM cyclohexane-1,2-diaminetetra-acetic acid, 10 % glycerol, 2 mM DTT and 1 % Triton X-100. Cell lysates were separated by SDS/PAGE (15 % gel), and expression of endogenous GSTP and GAPDH was detected by Western blotting.

RESULTS

Activity and expression profiles of HATs during hepatocarcinogenesis

To evaluate the activity and expression profiles of HATs during hepatocarcinogenesis, we performed chemical carcinogenesis in the rat liver in accordance with the Solt–Farber protocol [37]. This model of liver chemical carcinogenesis is a widely used system for the study of molecular and cellular processes leading to cancer. In this protocol, rats were fed a combination of DEN and AAF and then underwent PH. At the end of 7 weeks, the livers had large numerous hyperplastic nodules, and the rats were killed (Figure 1A). We prepared four types of control experiments: rats underwent saline injection; were injected with DEN; underwent AAF feeding; underwent PH but were not treated with DEN and AAF. We checked the reproducibility of the carcinogenic experiments. Western blotting analysis of the cytosol fractions with an anti-GSTP antibody revealed that GSTP was induced at 7 weeks after combined treatment with DEN, AAF and PH, but no GSTP was detected in any of the control rats (Figure 1B).

We first investigated the HAT activity of nuclear extracts during hepatocarcinogenesis. The assay using core histones or nucleosome histones as substrates revealed that HAT activity in livers with hyperplastic nodules was indistinguishable from that in control rat livers (results not shown). For determination of the expression profiles of HATs during hepatocarcinogenesis, we performed Western blot analysis using nuclear extracts and specific antibodies to each of the HATs (Figure 1B). The HATs best characterized as transcriptional co-activators are p300, CBP, P/CAF and GCN5. The expression levels of P/CAF and GCN5 showed no change during hepatocarcinogenesis, but expression of both p300 and CBP decreased. Next, we observed the expression levels of the MYST-type acetyltransferases, which are involved in a wide range of regulatory functions [1]. Expression of TIP60 was unchanged during hepatocarcinogenesis, whereas MOZ expression increased. MORF was not detected (results not shown). Among those we assayed, MOZ was the sole HAT whose expression was positively correlated with GSTP expression during hepatocarcinogenesis.

Induction of the intact form of MOZ during hepatocarcinogenesis

MOZ belongs to the MYST family of HATs and frequently is rearranged in leukaemia [10]. MOZ fusion partners include CBP, p300 and TIF2 (transcriptional intermediary factor 2); all of these proteins are also known to be transcriptional co-activators [10,24–26]. MOZ is a transcriptional regulator in haemopoiesis, and MOZ fusion proteins antagonize MOZ function and lead to leukaemogenesis [26,28]. Using Western blotting and RT (reverse transcriptase)–PCR analyses, we assessed whether MOZ was translocated and thus fused with these transcriptional co-activators during hepatocarcinogenesis (Figure 2 and results not shown). We generated specific antibodies against the N- and C-terminal regions of rat MOZ, and we also used the anti-MYST antibody, which recognizes a motif (amino acids 671–685) in the MYST region of MOZ. These three antibodies recognize different parts of MOZ. Western blot analysis revealed that MOZ induced in livers with hyperplastic nodules and recognized by the three different antibodies were all the same size (Figure 2A), as

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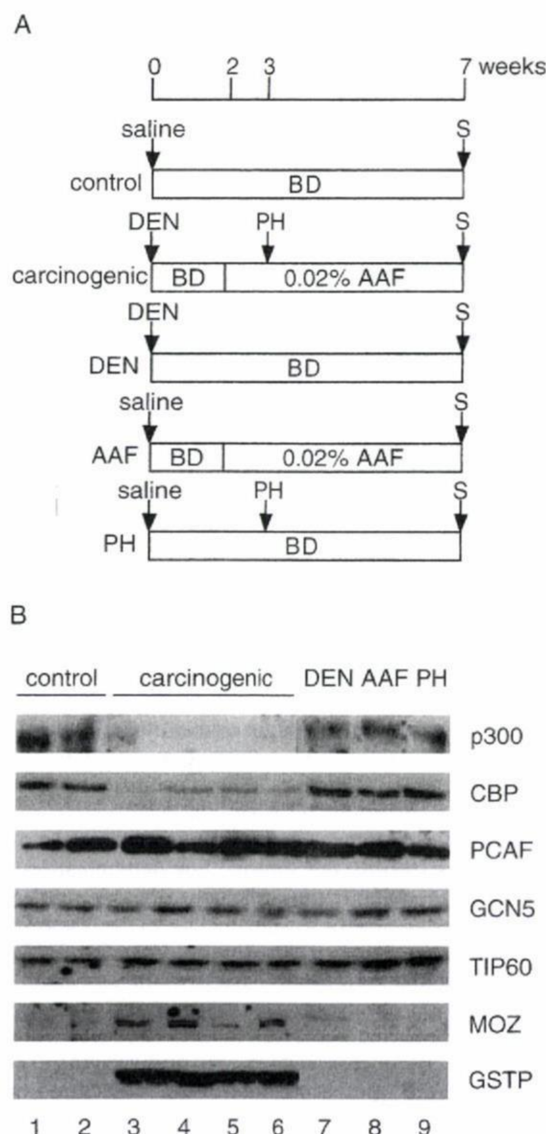

Figure 1 Expression profiles of HAT during hepatocarcinogenesis

(**A**) The Solt–Farber protocol for chemically induced hepatocarcinogenesis in rats [37]. BD, basal diet; S, times at which rats were killed. (**B**) Expression profiles of HATs were investigated in control livers and those with hyperplastic nodules. Nuclear extracts were prepared from livers of various rats, and immunoblot analysis was performed with specific antibodies, as described in the Experimental section. GSTP in the cytosol fraction was also detected (bottom). The fractions shown in lanes 1 and 2 were from control rats; lanes 3–6, rats having livers with hyperplastic nodules; lane 7, rat treated with DEN only; lane 8, rat treated with AAF only; lane 9, rat underwent PH only.

was the less-abundant MOZ in the control rat liver. We characterized additional fusion partners, including p300, CBP and TIF2. The sizes of these proteins in livers with hyperplastic nodules were the same as those in control livers (Figures 1B and 2B). These results suggested that the intact form of MOZ was induced and that translocation of MOZ did not occur during chemical hepatocarcinogenesis in rats. To confirm these results, we performed RT–PCR with three sets of primers spanning the MOZ regions in which rearrangement occurred frequently [10,24–26]. Sequencing of PCR products revealed that MOZ rearrangement did not occur during hepatocarcinogenesis (results not shown). We further examined the MOZ–CBP chimaeric transcript by hemi-nested PCR, but the amplification product was not detected

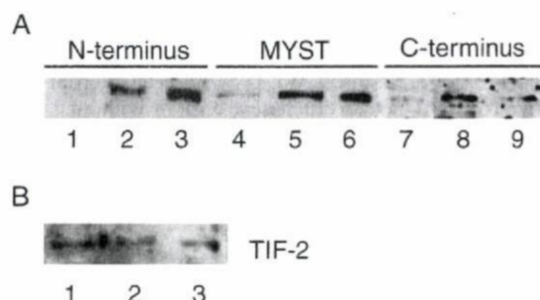

Figure 2 Induction of the intact form of MOZ during hepatocarcinogenesis

(**A**) Nuclear extracts were prepared from control (lanes 1, 4 and 7) and livers with hyperplastic nodules (lanes 2, 3, 5, 6, 8 and 9), separated by SDS/PAGE (7.5 % gel), and immunoblotted using polyclonal antibodies against the N- (lanes 1–3) or C- (lanes 7–9) terminal region of MOZ or the anti-MYST antibody (lanes 4–6). (**B**) The putative MOZ fusion partner, TIF-2, was detected with the anti-TIF-2 antibody. The fraction shown in lane 1 is from control; those in lanes 2 and 3 were from livers with hyperplastic.

(results not shown). These results indicate that the intact form of MOZ was induced and that MOZ translocation did not occur during the early stages of hepatocarcinogenesis.

Activation of GSTP promoter activity by MOZ through the GPE

MOZ functions as a transcriptional co-activator and participates as a mediator in haemopoiesis [28,42]. To characterize the effect of MOZ on hepatocarcinogenesis-specific gene expression, we asked whether exogenous MOZ would enhance GSTP promoter activity. GSTP is strongly and specifically expressed during chemical hepatocarcinogenesis and is considered to be an excellent tumour marker [29]. The transcriptional regulatory region of the rat GSTP gene includes enhancer and silencer elements [30,31]. To examine the effect of MOZ on GSTP promoter activity, −2.5GST-luciferase (which has the entire GSTP regulatory region and promoter) was co-transfected with MOZ expression plasmid or control empty vector into rat hepatoma H4IIE cells (Figure 3A). MOZ enhanced GSTP promoter activity (Figure 3B). Luciferase activity in the presence of various concentrations of MOZ was assayed, and MOZ demonstrated dose-dependent enhancement of GSTP promoter activity (Figure 3C). To more closely define the MOZ response element, we used two reporter plasmids: −2.15GST-luciferase, which lacked the GPE, and −91GST-luciferase, which lacked both the GPE and silencer regions (Figures 3A and 3B). These reporter plasmids were not transactivated, thereby suggesting that MOZ activates GSTP promoter activity through the GPE.

MOZ interacts with MafK both *in vitro* and *in vivo*

The GPE1 element in GPE is a key control element responsible for GSTP expression in preneoplastic tissue. GPE1 is similar in sequence to ARE (antioxidant-response-like element), MARE (Maf recognition element) and TRE [PMA ('TPA')-responsive element] [30,33,34]. A recent study showed that the Nrf2–MafK heterodimer binds to GPE1 and regulates GSTP promoter activity [34]. To determine the mechanism of the MOZ-associated enhancement of GSTP promoter activity, we tested whether MOZ could bind Nrf2 and MafK. We previously showed that MOZ interacted with c-Jun through the bZIP domain *in vitro* [39]; Nrf2 and MafK also have bZIP domains. To determine MOZ binding partners, we performed an *in vitro* pull-down assay using ^{35}S-labelled full-length MOZ. We fused the Nrf2 DNA-binding domain to maltose-binding protein, incubated it with ^{35}S-labelled MOZ, and precipitated it with amylose resin, but interaction

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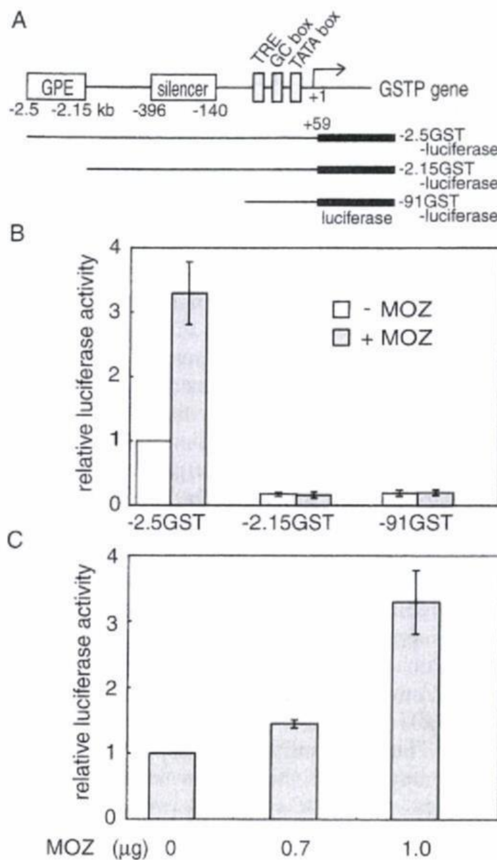

Figure 3 MOZ activates the GSTP promoter activity through the GPE

(**A**) Diagram of the 5′-flanking region of the rat GSTP gene and the reporter constructs for observing the effect of MOZ on the promoter activity of the GSTP gene. (**B**) We co-transfected 100 ng of the reporter plasmid with (grey columns) or without (white columns) 1 μg of MOZ expression plasmid (pCI-MOZ) into H4IIE rat hepatoma cells. All transfection assays were repeated at least three times. The relative luciferase activity was calculated from mean values relative to the activity of −2.5GST-luciferase in the absence of MOZ. Each error bar indicates ±S.D. (**C**) Dose-dependent transactivation of −2.5GST-luciferase by MOZ. Relative luciferase activities are shown as in (**B**).

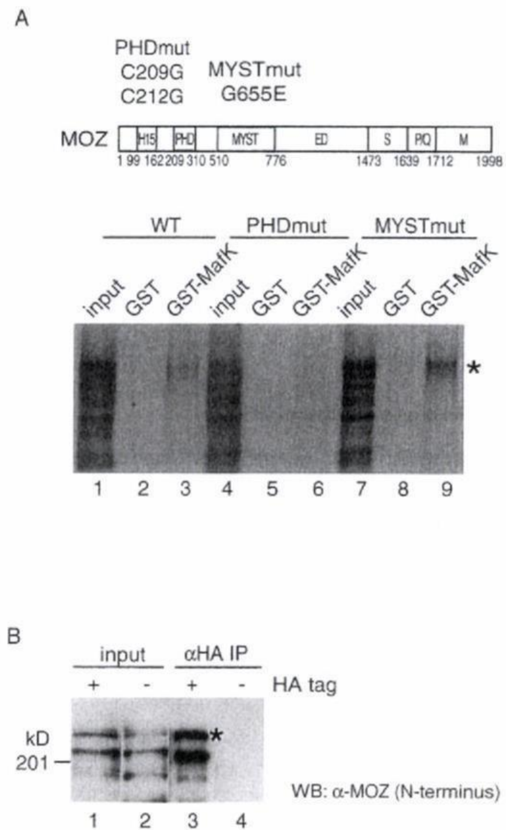

Figure 4 MOZ interacts with MafK *in vitro* and *in vivo*

(**A**) Structural domains of MOZ were indicated as follows: H15, histones H1- and H5-like module; MYST, MYST acetyltransferase domain; ED, glutamic acid/aspartic acid-rich acidic regions; S, serine-rich domain; P/Q, proline/glutamine-stretch; and M, methionine-rich domain. Also shown are the mutation positions in the PHD finger and MYST regions. Indicated wild-type and mutated *in vitro*-translated [^{35}S]MOZ proteins were incubated with GST (lanes 2, 5 and 8) or GST–MafK (lanes 3, 6 and 9). MOZ protein retained on the GST-conjugated beads after extensive washing was analysed by SDS/PAGE and autoradiography. The amount of input (lanes 1, 4 and 7) is equivalent to 10 % of the reaction volume in the assay. [^{35}S]MOZ proteins are indicated by asterisks (*). (**B**) MOZ expression plasmid was co-transfected with HA-tagged MafK (lanes 1 and 3) or non-tagged MafK (lanes 2 and 4) into HeLa cells, and nuclear extracts were prepared. Immunoprecipitation (IP) experiments were performed with anti-HA antibody. Immunoprecipitates (lanes 3 and 4) and 5% of input (lanes 1 and 2) were resolved by SDS/PAGE (7.5 % gel) and detected by Western blotting using anti-N-terminal MOZ antibody. MOZ proteins are indicated by asterisks (*).

between MOZ and the Nrf2 DNA-binding domain was not detected (results not shown). Next, we evaluated the interaction between MOZ and MafK using a GST pull-down assay and found that ^{35}S-labelled MOZ interacted with GST–MafK but not with GST alone (Figure 4A, lanes 1–3). Unique structural domains are identified in MOZ [28]. To identify the region required for the interaction between MOZ and MafK, two MOZ derivatives with double and single point mutations in the PHD zinc-finger (C209G and C212G) and the MYST (G655E) regions respectively, were generated. The PHD zinc-finger and the MYST regions are important for binding to specific nuclear protein partners and HAT activity, respectively [43,44]. The mutant in the PHD zinc-finger region was not able to interact with GST–MafK, whereas the mutation in the putative acetyl-CoA-binding site in the MYST region did not affect the binding to MafK (Figure 4A, lanes 4–9). These results suggest that MOZ interacts with MafK in the absence of the heterodimer partner, Nrf2, mediated by the PHD zinc-finger region of MOZ.

To evaluate the interaction between MOZ and MafK under physiological conditions, we attempted to detect immunoprecipitated MOZ, but endogenous MOZ in nuclear extracts from H4IIE and HeLa cells could not be detected. Therefore we next introduced the MOZ expression plasmid with HA-tagged or non-tagged MafK into HeLa cells, and nuclear extracts were prepared. MOZ was immunoprecipitated only in nuclear extracts expressing HA-tagged MafK (Figure 4B). Some degraded MOZ proteins were detected in nuclear extracts and these proteins were also immunoprecipitated. GST pull-down and immunoprecipitation experiments suggest that MOZ may interact with the MafK moiety of the Nrf2–MafK heterodimer *in vivo*.

MOZ functions as a co-activator of the Nrf2–MafK heterodimer

MOZ preferentially interacted with MafK and up-regulated GSTP promoter activity through GPE, which contains the binding site for the Nrf2–MafK in the reporter assay (Figures 3 and 4). These data suggest that MOZ is a potential co-activator of Nrf2–MafK heterodimer. To test this hypothesis, we investigated whether MOZ could stimulate Nrf2–MafK-mediated transactivation (Figure 5). We have previously reported that Nrf2 simulates GPE1-mediated transactivation in F9 cells, which are considered to lack AP1 (activator protein 1) activity and to express excess amounts of small Maf proteins, including MafK [34]. MOZ or Nrf2

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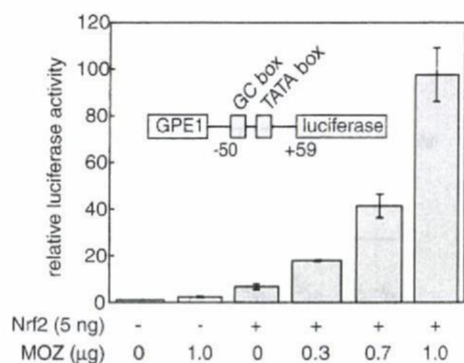

Figure 5 MOZ is a co-activator of Nrf2

Nrf2-mediated transactivation by MOZ was examined in mouse embryonic carcinoma F9 cells. We co-transfected 100 ng of the reporter plasmid (GPE1-luciferase, in the panel) and 5 ng of *Renilla* luciferase plasmid (phRL-tk) with 0, 0.3, 0.7 and 1 μg MOZ expression plasmid (pCI-MOZ) in the absence (−) or presence (+) of Nrf2 expression plasmid (pAβ2-Nrf2, 5 ng). The luciferase activity was normalized to *Renilla* luciferase activity. Relative luciferase activity was calculated from the mean values relative to the activity of GPE1-luciferase without Nrf2 and MOZ. Each error bar indicates $\pm$S.D.

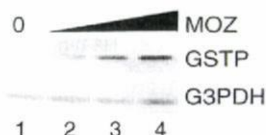

Figure 6 MOZ induces endogenous GSTP expression

H4IIE cells were transfected with 0, 0.4, 0.7 and 1.0 μg of MOZ expression plasmid (pCI-MOZ, lanes 1–4), and cell lysates were prepared. Endogenous GSTP and GAPDH ('G3PDH') were detected by immunoblotting.

expression plasmid was co-transfected with the reporter plasmid GPE1-luciferase (which includes GPE1 and the GSTP promoter) into F9 cells. MOZ and Nrf2 slightly enhanced the activity of the reporter construct. As expected, MOZ, when in the presence of Nrf2, dose-dependently stimulated GPE1-mediated GSTP promoter activity. MOZ did not stimulate the promoter activity of reporter plasmids including the mutated Nrf2 binding site (results not shown).

Induction of endogenous GSTP expression by MOZ in rat hepatoma cells

As we described above, MOZ stimulated the GSTP promoter activity mediated by Nrf2–MafK. We then assessed the effects of MOZ overexpression on GSTP expression in H4IIE cells. Transiently overexpressed MOZ induced expression of endogenous GSTP but not GAPDH (Figure 6). The induction of GSTP protein was dependent on the exogenous MOZ expression. These results suggest that MOZ functions as a co-activator of the Nrf2–MafK heterodimer and may stimulate GSTP gene expression during hepatocarcinogenesis.

DISCUSSION

HATs contribute to tumour suppression, and loss or dysregulation of these activities may be linked to tumorigenesis [45]. To gain insight into the roles of HATs in liver cancer, we analysed the expression profiles of HATs during hepatocarcinogenesis and evaluated their roles in hepatocarcinogenic-specific gene expression.

We have shown that MOZ expression was up-regulated during hepatocarcinogenesis. MOZ functions as a co-activator of AML1-mediated transcription, and the AML1–MOZ complex might play a role in cell differentiation [28,42]. MOZ frequently is rearranged in leukaemia, and the MOZ fusion protein antagonizes MOZ function in haemopoiesis [26,28]. Even though MOZ rearrangement does not occur during hepatocarcinogenesis, we documented an anomalous increase of MOZ. Because HATs regulate global gene expression [1,46], dysregulation of MOZ may induce unusual gene expression, leading to hepatocarcinogenesis.

Recently, the MOZ–MORF complex including BRPF (bromodomain- and PHD finger-containing) 1/2/3 paralogue and ING5 (inhibitor of growth 5; tumour suppressor) was purified [47]. MORF was not detected in nuclear extracts from livers with hyperplastic nodules (results not shown), so that MOZ, but not MORF, complex may regulate GSTP expression. ING5 is also included in HBO1 [HAT binding to ORC1 (origin recognition complex subunit 1)] complex. Interestingly, ING4, another member of ING family proteins, exists in HBO1 complex, but not MOZ complex. AML1-dependent promoter activity is stimulated by ING5, but not ING4 [47]. This raises a possibility that overexpressed MOZ may affect regulation of AML1-dependent gene expression. ING5 tumour suppressor is included in both HBO1 and MOZ complexes, which are important for DNA synthesis [47]. Overexpressed MOZ might trap ING5 and generate partial complexes, and further, HBO1 complex would be affected with the change of ING5 level. Thus aberrantly expressed MOZ during hepatocarcinogenesis may disturb the tumour suppressor function of ING5 complexes and DNA synthesis, which lead to tumorigenesis.

We also found that the expression of p300 and CBP were decreased during hepatocarcinogenesis. Although AAF blocks the proliferation of hepatocytes, GSTP-expressing cells escape from the growth inhibition and continuously grow in the Solt–Farber model. Trautwein et al. [48] reported that AAF blocks cell-cycle progression after PH by inducing the cyclin-dependent kinase inhibitor p21. Expression of p21 is regulated mainly by the tumour-suppressor protein p53, and full transcriptional activity of p53 requires the co-activators p300/CBP [49–51]. Down-regulation of p300 and CBP reduces p53 activity and leads to cell-cycle progression of GSTP-expressing cells, suggesting that p300 and CBP may be considered tumour suppressors, and their loss of function may be a link to hepatocarcinogenesis.

GSTP is a Phase II detoxification enzyme involved in the metabolism of carcinogens, and it plays a protective role during chemical hepatocarcinogenesis [52]. The Nrf2–MafK heterodimer is important for the GSTP expression during early hepatocarcinogenesis, but it is difficult to explain the markedly increased expression of GSTP in livers with hyperplastic nodules solely on the basis of the increased quantity of Nrf2 [34]. We found that the expression of MOZ was well correlated with GSTP expression during hepatocarcinogenesis; MOZ also functioned as a co-activator of the Nrf2–MafK heterodimer. We reported that the binding activity of Nrf2–MafK heterodimer to GPE1 is much stronger than that of MafK homodimer. Further Nrf2 alone could not bind to GPE1, and the Nrf2 mRNA level is increased in cells from hyperplasic nodules when compared with those from normal livers [34]. Histones H3 and H4 are acetylated in both GPE1 and in the promoter regions of the GSTP gene in the H4IIE hepatoma cell line but not normal liver [34]. This acetylation coincides with the activation of GSTP expression. MOZ may contribute acetylation of histones in the regulatory region of the GSTP gene. Elevation of both MOZ and Nrf2 expression may be required for the dramatically increased gene expression of GSTP observed during hepatocarcinogenesis *in vivo*. To understand

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the molecular mechanism of the GSTP induction mediated by Nrf2–MafK heterodimer and MOZ, we proceeded to identify the regions of MOZ and MafK required for the GSTP expression in exogenously Nrf2-expressed H4IIE cells.

The activation mechanism of GSTP expression is classified into two types: specific induction in livers with hyperplastic nodules by chemical carcinogens, and non-specific induction by non-carcinogenic agents such as antioxidants [29,53]. The former induction may require both Nrf2 and MOZ, but only Nrf2 may be necessary for the latter. Preneoplastic foci and nodules are derived from GSTP-positive single cells [54]. The mechanism of the generation of the GSTP-positive single cell is unclear, and specific induction of GSTP has not been reproduced in cell lines by using chemical carcinogens. The use of transgenic or MOZ knockout animals would probably enable us to demonstrate the mechanism of chemical carcinogen-associated GSTP induction during hepatocarcinogenesis.

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Inhibition of estrogen action by 2-phenylchromone as AhR agonist in MCF-7 cells

Joohee Jung [1], Kunie Ishida, Jun-ichi Nishikawa, Tsutomu Nishihara *

Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan

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Abstract

Large amounts of phytoestrogen, a group of estrogen derived from plant sources, are taken from the diet by Asians, but a sign of feminization has not been fully recognized. In this study, we found that some flavonoids inhibited an effect on estrogen action without estrogen receptor (ER) binding. Considering the report that dioxin, an aryl hydrocarbon receptor (AhR) agonist, disrupts the transcriptional activity of ER without binding to the ER, 14 flavonoids were examined for the transcriptional activity of AhR by the yeast reporter assay (AhR). Among them, 2-phenylchromone (flavone, FLA) showed the highest activity. FLA increased the expression of CYP1A1 mRNA, and inhibited the expression of progesterone receptor and pS2 mRNA in MCF-7 cells via non-ER-mediated pathway. Further studies showed that FLA had agonist activity for AhR and enhanced the proteosome-dependent degradation of ERα protein. Thus, FLA inhibited the estrogen action without binding to the ER by acting as a competitive agonist for AhR, which meaning that there can be anti-estrogenic flavonoids such as FLA as well as estrogenic ones such as isoflavones.
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Keywords: 2-phenylchromone; Aryl hydrocarbon receptor; Estrogen receptor; MCF-7 cell

Introduction

Flavonoids are widely present in plants, and possess diverse physiological activities. Numerous reports have implicated flavonoid phytochemicals as possessing hormone-disrupting activity (Diel et al., 2004; Kuo, 2002; Hsieh et al., 1998). Similar to estrogen's role, several of these phytochemicals have been shown to prevent osteoporosis and cardiovascular disease (Lee et al., 2007; Bingham et al., 1998; Humfrey, 1988; Kutzer and Xu, 1997).

Estrogen plays important roles in the function, growth and differentiation of the mammary gland, uterus, and ovary. It also affects other tissues, including the bone, liver, cardiovascular system, and brain. Estrogen acts primarily through the estrogen receptor (ER), which is a member of the nuclear hormone receptor superfamily and a ligand-dependent transcription factor (Evans, 1988; Speroff, 2000). The biological activities of many flavonoids may occur via the ER-mediated pathway. It is well known that soybean and its products contain isoflavones, such as genistein, coumesterol, and diadzein, and Asians including Japanese have taken a large amount of such phytoestrogens from food for several hundreds of years. Nishikawa (2003) estimated that Japanese took phytoestrogen at 15 mg/day, corresponding to about ten times the tolerable daily intake of β-estradiol (E2). Nevertheless, feminine qualities in man did not appear (Iwamoto et al., 2006). It may be due to intake of food containing phytoestrogen along with anti-estrogenic substances. The effect of flavonoids on estrogen action by the ER-mediated pathway has been investigated through the authors (Nishihara et al., 2000), but the elucidation of the action of flavonoids is scant. Some chemicals may bind to other receptors, such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Brosens and Parker, 2003; Mikamo et al., 2003). Among them, we had an interest in

* Corresponding author. Present address: School of Pharmacy, Hyogo University of Health Sciences, 3-6, Minatojima-1, Chuo, Kobe, Hyogo 650-8530, Japan. Tel.: +81 78 304 3000; fax: +81 78 304 2700.

E-mail address: nishihara@huhs.ac.jp (T. Nishihara).

[1] Present address: Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan.

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AhR, which is a ligand-activated transcription factor that stimulates gene expression when coupled with AhR nuclear translocator (ARNT) (Carver and Bradfield, 1997; Denison and Whitlock, 1995). TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a ligand of AhR) induces CYP1A1, CYP1A2, and CYP1B1 that hydroxylate E2. Moreover, TCDD and other agonists for AhR disrupt the transcriptional activity of ER by degradation of ER (Buchanan et al., 2000, 2002). Thus, agonists of AhR may mimic the effects of estrogen through the mechanism that is involved in the degradation of ER by a transcriptional active AhR–ARNT complex (Ohtake et al., 2003).

In this study, we investigated the effect of flavonoids on AhR and ER action (Table 1), and found that in MCF-7 cells, 2-phenylchromone (FLA, the structurally most basic compound) inhibited the expression of ER target genes, suggesting antagonist activity of ERα expressed via AhR in an indirect manner.

Table 1
Structures of flavonoids used in this study

Structures	Classifications	Test chemicals
	Flavones	Flavone (FLA)
	Flavonols	Kaempferol Quercetin
	Isoflavones	Biochanin A Daidzein Genistein
	Flavanones	Hesperetin Hesperidin Naringenin
	Chalcones	Phloretin
	Coumarins	Bromofluoro coumarin 2-Bromomethyl-7-methoxycoumarin Coumestrol 4-Methylumbeliferone

Materials and methods

Chemicals

FLA, E2, kaempferol, hesperidin, and α- and β-naphthoflavone (α-NF and β-NF) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), hesperetin and bromofluoro coumarin were from Extrasynthese (Genay Cedex, France), 2-bromomethyl-7-methoxycoumarin was from BD Gentest (CA, USA), 4-methylumbeliferone was from Sigma-Aldrich Co. (MO, USA), and MG-132 was from Calbiochem (Darmstadt, Germany). Biochanin A, coumestrol, daidzein, genistein, naringenin, phloretin, and quercetin were provided by Dr. H. Utsumi (Kyushu University).

Yeast assay for AhR ligand activity

The yeast transformed with the AhR–ARNT complex and xenobiotic-responsive element (XRE) plasmids was used as described by Miller (Miller, 1999). The AhR ligand activity was determined essentially according to the method of Adachi et al. (2001). The yeast strain YCM3 was grown for 5 h at 30 °C in SD medium lacking tryptophan. Test chemicals were added at given concentrations to 5 µl of culture and 200 µl of SD medium containing 2% galactosidase and incubated overnight at 30 °C. After the cell density was determined by reading O.D. at 595 nm, 10 µl of cell suspension was added to 140 µl of Z-buffer and β-galactosidase activity was determined by *o*-nitrophenol-β-D-galactopyranoside for 60 min at 37 °C. Absorbance was read at 415 nm.

Cells

MCF-7 cells were grown for routine maintenance in Eagle's minimal essential medium (EMEM) with phenol red (Nissui Pharmaceuticals Co., Tokyo, Japan), supplemented with 10 mM non-essential amino acids (Nacalai Tesque Co., Tokyo, Japan) and 10% dextran-charcoal treated fetal bovine serum (FBS). Cells were maintained in a humidified environment at 37 °C with 5% CO_2 in air.

RNA isolation and RT-PCR

MCF-7 (4×10^5 cells/ml) cells were plated in 35-mm dishes and, after 48 h incubation, treated with chemicals for 24 h. After treatment, the cells were washed twice with PBS and RNA was then isolated using Trizol (Invitrogen, CA). cDNA synthesized from 0.8 µg of total RNA using ReverTra Ace-α™ (TOYOBO, Osaka, Japan) and PCR for progesterone receptor (PR), pS2, CYP1A1, and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) was performed using Ampli Taq (Roche, Basel, Switzerland). The optimum cycle number that fell within the exponential range of response was used for PR (30 cycles), pS2 (21 cycles), CYP1A1 (30 cycles), or G3PDH (17 cycles).

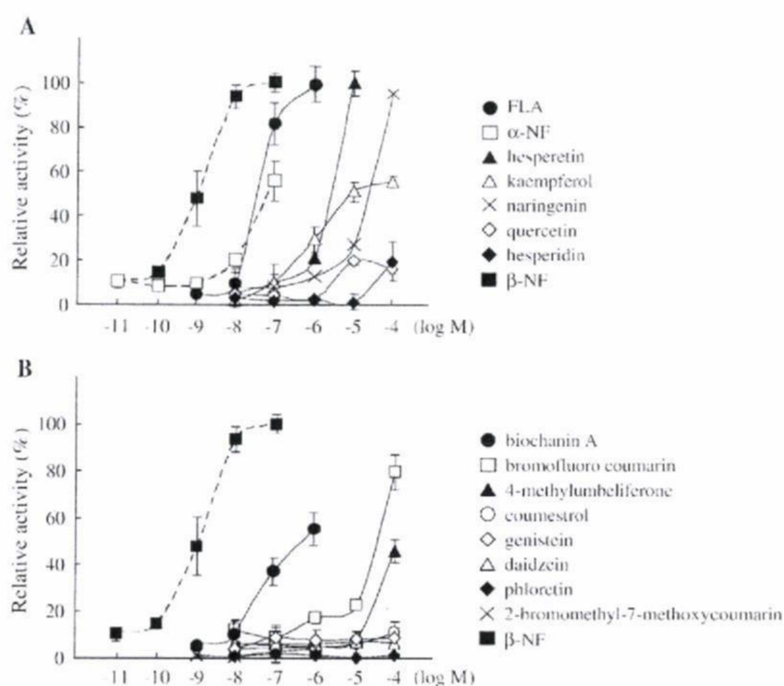

Fig. 1. AhR agonist activity of flavonoids. The test chemicals were applied to the recombinant yeast assay for AhR ligand activity as described in Materials and methods. The test chemicals were divided into two groups; a group included flavones, flavonols, and flavanones (A), and the other group included isoflavones, chalcones, and coumarins (B). Some flavonoids including FLA showed positive activity.

Yeast two-hybrid assay (ER)

We used a yeast two-hybrid assay system with the rat ER (rER) α and the coactivator, TIF2 as described in earlier works (Nishihara et al., 2000; Jung et al., 2004).

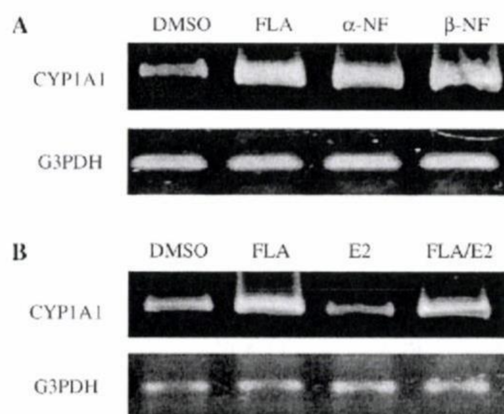

Fig. 2. Effect of FLA on the expression of CYP1A1 mRNA in MCF-7 cells. FLA (10 μM) was incubated with MCF-7 cells for 24 h in the absence (A) and presence (B) of E2 (10 pM). α- and -β NFs (10 μM) were used as positive controls, and DMSO as negative control. The expression of CYP1A1 and G3PDH mRNA (as an internal control) was detected by RT-PCR as described in Materials and Methods. FLA induced the expression as well as α-, β-NFs (A), but it was inhibited by E2 (B).

Estrogen receptor competitive binding assay

The binding activity of chemicals to human ER (hER) α was determined using a fluorescence polarization assay by FP Screen-for-Competitors Kit (ERα, high sensitivity; PanVera, Madison, WI). Briefly, 1 μl of each chemical solution was added to 49 μl of screening buffer in tubes and mixed well by shaking. Then, 50 μl of ERα-fluorescence estrogen (ES1) complex solution was added to the tube, incubated at room temperature for 1 h, and the fluorescence was determined using BEACON2000 (PanVera, Madison, WI). DMSO (0% inhibition) instead of the chemical solution was used as a negative control and 10 μl of ES1 (50 nM) instead of ERα ES1 complex as a

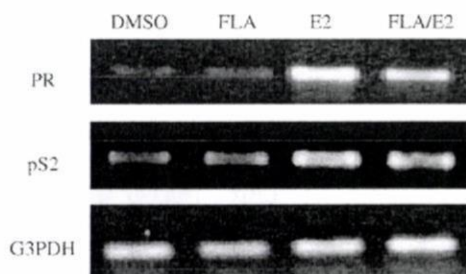

Fig. 3. Effect of FLA on mRNA level of E2-dependent target genes. FLA (10 μM) was incubated with MCF-7 cells for 24 h in the absence and presence of E2 (10 pM). The expression of PR, pS2 and G3PDH (as an internal control) mRNA was detected by RT-PCR. FLA repressed the expression by E2.

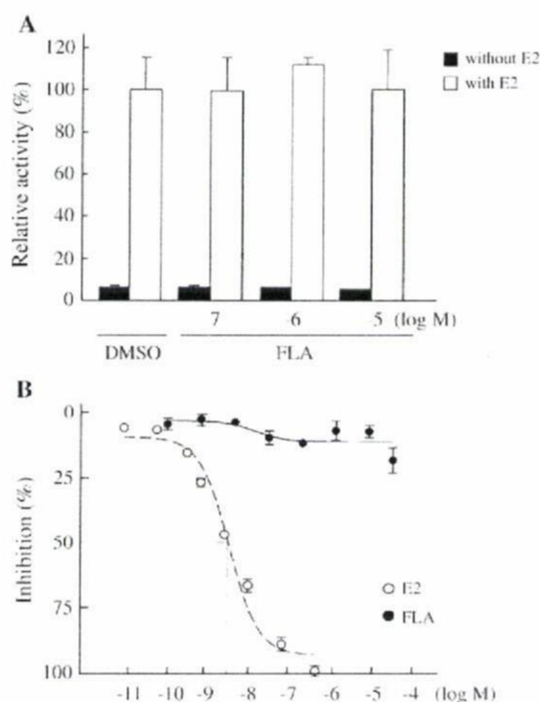

Fig. 4. Estrogen activity of FLA. (A) ERα agonist or antagonist activity of FLA was examined by yeast two-hybrid assay, and the relative activity (%) was calculated as the percentage of E2 (5 nM) activity (100%). (B) Binding affinity of FLA was examined by ERα competitive binding assay as described in Materials and Methods. FLA exhibited no effect on estrogen binding activity to ERα.

positive control (100% inhibition). Curve fitting was performed using GraphPad Prism 2.01 software to obtain IC_{50}.

Protein isolation and Western blots

MCF-7 (4×10^5 cells/ml) cells were plated in 35-mm dishes and, after 48 h, treated with chemicals for the indicated times. After treatment, the cells were washed twice with PBS and then lysed in 70 μl of lysis buffer containing 8 M urea, 1% NP-40, and 2% 2-mercaptoethanol. After removing the cell debris, the supernatants were used for protein concentration assay. The protein was boiled for 2 min, resolved on a 10% SDS-polyacrylamide gel, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore immobilon transfer membrane, MA). After the membrane was blocked using 3% skimmed milk (Yukijirushi, Tokyo, Japan) overnight at 4 °C, it was probed with primary antibodies ERα (1:200 in 1% skimmed milk, Santa Cruz Biotechnology Inc., CA) and G3PDH monoclonal antibodies (1:1000 in 1% skimmed milk, Chemicon International, MA). Following incubation with peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (Amersham Pharmacia Biotech, UK).

Results and discussion

In the yeast reporter gene assay, the effect of flavonoids on AhR was evaluated because it had been reported that TCDD and other AhR agonists inhibited the expression of several E2-induced genes without binding to ER (Buchanan et al., 2002). Among 14 tested flavonoids, 7 compounds (FLA, biochanin A, hesperitin, kaempferol, naringenin, bromofluoro coumarin and 4-methylumbeliferone) dose-dependently increased the AhR activity (Fig. 1A and B). On the contrary, Hamada et al. (2006) reported that some of these flavonoids suppressed TCDD-induced CYP1A1 expression in dioxin-responsive HepG2 cells by permeating Caco-2 cell monolayers. The difference may involve the metabolism of flavonoids by drug-metabolizing enzymes in the Caco-2 cells. Furthermore, we investigated FLA, which was particularly responsible for the high activity of AhR. Although FLA was ten times weaker than β-NF (positive control), it was stronger than the other flavonoids and α-NF (a second positive control). Therefore, FLA was chosen for further study. AhR agonists induce the expression of several genes. For example, mRNA levels of CYP1A1 are induced by TCDD and other AhR ligands (Whitlock et al., 1996). RT-PCR assay indicated that in MCF-7 cells, FLA (fold, 2.6 ± 1.0) induced the expression of CYP1A1 mRNA at similar levels to α- and β-NF (folds, 2.2 ± 0.5 and 2.4 ± 0.5) (Fig. 2A). In contrast, E2 inhibits the expression of genes induced by AhR ligands (Stacey et al., 1999). In this study, the induction of CYP1A1 mRNA by FLA (fold, 2.2 ± 0.1) was inhibited in combination with E2 (fold, 1.5 ± 0.4) (Fig. 2B). The expression of G3PDH mRNA was measured as control, and then it was not changed. Thus, the results suggest that FLA acted as an AhR agonist.

When we tested the anti-estrogenic activity of many chemicals including flavonoids by the yeast two-hybrid assay and the competitive ER binding assay, some flavonoids and

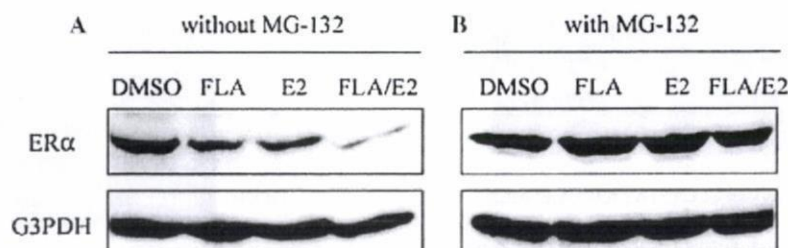

Fig. 5. Effect of FLA on ERα protein level. In the absence (A) or presence (B) of MG-132 (protease inhibitor), FLA (10 μM) was incubated with/without E2 (10 pM) in MCF-7 cells for 24 h. The cell lysates were subjected to SDS-PAGE and Western blot analysis for ERα and G3PDH (as an internal control). FLA induced the degradation of ERα and it was prevented by MG-132.

their metabolites were determined to be ER antagonists as well as agonists (Nishihara et al., 2000; Ahn et al., 2004a,b; Okamoto et al., 2006). As mentioned earlier in this work, however, these assays are insufficient to explain the anti-estrogenic activity of some flavonoids because these assays can measure only when chemicals directly affect the interaction with ERα. When we screened for the active compound by the reporter gene assay using MCF-7 cells in the presence of E2, some chemicals inhibited the transcriptional activity of ER (details not shown). The expressions of PR and pS2 mRNA were induced by E2 depending on the dose, indicating that these expressions demonstrated estrogen activity (Seo et al., 2003; Petz et al., 2002; Kim et al., 2000). As an AhR agonist, FLA was investigated for estrogen action. E2 (1 pM) induces the mRNA expression of PR (fold, 9.6 ± 1.2) and pS2 (fold, 6.3 ± 0.1) in MCF-7 cells. The E2 induced mRNA expressions of PR (fold, 2.1 ± 0.2) and of pS2 (fold, 1.5 ± 0.1) was minimized by treating with FLA, although FLA alone did not affect these expressions (Fig. 3). The expression of G3PDH mRNA was measured as control. To reconfirm the mode of action of FLA on ERα, the binding activity was examined. As shown in Fig. 4A, FLA had neither the agonistic, nor antagonistic activity on ER in a yeast two-hybrid assay using rER. In the competitive binding assay using hER, FLA did not inhibit binding of ER (Fig. 4B). The results suggested that FLA of the basic structure had anti-estrogenic activity without binding to the ER receptor, though several derivatives of the flavone group have estrogenic activity (Innocenti et al., 2007; Hiremath et al., 2000).

AhR agonists induce rapid proteosome-dependent degradation of ER (Wormke et al., 2003). Furthermore, in breast cancer cells, ligand-bound AhR enhances ubiquitinated forms of ERα and proteasome-dependent degradation of ERα to repress the E2-induced transactivation (Wormke et al., 2000). As shown Figs. 1 and 2, FLA was suggested to be a ligand of AhR. Consequently, the protein level of ERα in the presence of FLA was determined in MCF-7 cells. FLA or E2 significantly decreased ERα protein level and FLA together with E2 enhanced this effect. Since TCDD, a ligand of AhR, activates proteasome-dependent degradation of ERα (Ohtake et al., 2003), the effect of protease inhibitor, a MG-132, was determined on ERα protein level. The results show that MG-132 prevented ERα from degradation by FLA (Fig. 5). Moreover, the results indicate that FLA induced the expression of CYP1A1 mRNA to enhance the degradation of ERα protein, and inhibited the expression of PR and pS2 mRNA through the AhR pathway.

It has been reported that some flavones show estrogenic activity through ER binding so that their intake has a preventive effect against prostate cancer (Raschke et al., 2006) and menopausal syndrome (Miller-Martini et al., 2001), and also has a stimulative effect on endometritis (Cline et al., 2004). Recently the Food Safety Commission of Japan published the upper-limit dose for soy isoflavone supplement to be 30 mg/day. Although Wood et al. (2006) reported that soy isoflavones had anti-estrogenic effects in the postmenopausal breast through ER signaling, we have presented in this work that FLA can be anti-estrogenic via AhR in MCF-7 cells. This means that other AhR agonists in food may potentially affect the action of estrogen.

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〈論 文—Original Paper〉

PRTR化学物質の各種核内受容体に対する結合性

井上大介* 松井久恵* 清　和成*
楊　敏** 胡　建英*** 荒金　淳†
廣辻淳二† 西川淳一†† 池　道彦*

Binding Affinity of PRTR Chemicals to Various Human Nuclear Receptors

Daisuke INOUE*, Hisae MATSUI*, Kazunari SEI*,
Min YANG**, Jianying HU***, Jun ARAGANE†,
Junji HIROTSUJI†, Jun-ichi NISHIKAWA†† and Michihiko IKE*

* Division of Sustainable Energy and Environmental Engineering, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

** State Key Lab of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

*** College of Environmental Science, Peking University, Beijing 100871, China

† Advanced Technology R&D Center, Mitsubishi Electric Co., 8-1-1 Tsukaguchi-Honmachi, Amagasaki, Hyogo 661-8661, Japan

†† Department of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

Abstract

Since the 1990s, population decreases, reproductive anomalies and malformations of highly aquatic animals have been increasingly reported. One possible cause is considered to be endocrine disruptive effects induced by environmental contaminants through a direct interaction with nuclear receptors, not only with steroid hormone receptors but also with other ones. In this study, we examined the binding affinities of 20 chemicals, which are registered in the Japanese Pollutant Release and Transfer Register (PRTR) and have been abundantly discharged into aquatic environments to eight human nuclear receptors and assessed their potential endocrine disruptive effects. Of the 20 PRTR chemicals tested, nonylphenol diethoxylate, telephthalic acid (TPA), and linear dodecyl-benzensulfonate (DBS) bound to at least two receptors at high concentrations. TPA and DBS enhanced the activities of both retinoic acid receptor (RAR) γ and vitamin D receptor (VDR) in a dose-dependent manner. This suggests that TPA and DBS may disturb the vitamin D endocrine functions mediated by a VDR-VDR homodimer or a VDR-RAR heterodimer. Also, our results indicate that endocrine disruptors unsuspected under the current assessment criteria could potentially bind to various nuclear receptors and disrupt endocrine systems mediated by such receptors.

Key words: aquatic environment, endocrine disruptive effect, nuclear receptor, PRTR chemical

1. はじめに

1990年代から，魚類や両生類など，水への依存度の高い野生生物種において，個体数の減少，生殖異常，形態異常の発生が数多く報告されている[1-6]。このような危機的状況をもたらした原因の一つは，環境中に放出された人工化学物質のホルモン様作用に起因する正常な内分泌バランスの攪乱にあるとされている[7]。内分泌攪乱化学物質（endocrine disruptors ; EDs）の内分泌機能への影響発現には，核内受容体（nuclear receptor ; NR）を介するメカニズムと介さないメカニズムが存在するが，その大部分はNRへの直接作用によると考えられている[8]。NRの中には，ヒトを含め，異なる生物種間で高い保存性を示すものもあることから[9]，EDsによる内分泌機能攪乱に伴う悪影響

＊ 大阪大学大学院工学研究科環境・エネルギー工学専攻 〒565-0871 大阪府吹田市山田丘2-1
＊＊ 中国科学院生態環境研究中心 Beijing 100085, China
＊＊＊ 北京大学環境学院 Beijing 100871, China
† 三菱電機㈱先端技術総合研究所 〒661-8661 兵庫県尼崎市塚口本町8-1-1
†† 大阪大学大学院薬学研究科 〒565-0871 大阪府吹田市山田丘1-6

がヒトにも生じる可能性があるものと推測される。

従来，EDsに関する研究は，主にエストロゲン受容体（estrogen receptor；ER）などの性ホルモン受容体や甲状腺ホルモン受容体（thyroid hormone receptor；TR）を中心に進められてきた。しかし近年，ヒトのNRファミリーに48種類の受容体が存在することが断定され[10]，EDsの作用点が性ホルモン受容体やTR以外のNRにもある可能性が議論されるようになった[11,12]。例えば，プラスチックの可塑剤等に使用されるフタル酸ジエステルの生体内代謝物であるフタル酸モノエステルによる雌生殖毒性等の生態毒性にはペルオキソーム増殖剤活性化受容体（peroxisome proliferator-activated receptor；PPAR）[13,14]が，また有機スズ化合物によるイボニシ貝のインポセックス発達の促進にはレチノイドX受容体（retinoid X receptor；RXR）[15]が関与していることが示唆されている。また，地下水汚染物質の一つ，トリクロロエチレンの生体内代謝物であるトリクロロ酢酸とジクロロ酢酸による肝臓癌とPPARαの関連性も指摘されている[16,17]。これらの新たな科学的事実から，化学物質の内分泌攪乱活性を評価するためには，様々なNRに対する作用を網羅的に検討することが重要であるといえる。すなわち，これまでに性ホルモン受容体やTRについて内分泌攪乱作用がないと判定された化学物質を含め，多くの人工化学物質のNRに対する作用を検討していくことが必要である。本研究では，水環境中に大量に放出されている化学物質のヒトNRに対する結合性を調査し，潜在的な内分泌攪乱作用の可能性を探った。被検物質には，環境汚染物質排出移動登録（pollutant release and transfer register；PRTR）制度の第1種指定化学物質に含まれ，公共用水域への年排出量が15tを上回る有機化学物質群の中から20種類を選定した。被検物質のNRに対する結合性の評価は，ヒトNRのうちERα，TRα，ビタミンA受容体（retinoic acid receptor；RAR）γ，RXRα，ビタミンD受容体（vitamin D receptor；VDR），PPARα/γ/δを対象として，*in vitro*でNRとコアクチベーターの相互作用を検出できるハイスループットスクリーニング法であるCoA-BAP（coactivator-bacterial alkaline phosphatase）法[18]を用いて実施した。

2．方　　法

2.1　化学物質

標準リガンドとして，ERαには17β-エストラジオール（E2），TRαには3,3',5-トリイオド-L-チロニン（T3），RARγには*all-trans*-レチノイン酸（retinoic acid；RA），RXRαには9-*cis* RA，VDRには1α,25-ジヒドロキシビタミンD3（$1,25(OH)_2D3$），PPARαにはGW7647，PPARγにはRosiglitazone，PPARδにはGW501516を用いた。

PRTR制度で第1種指定化学物質に指定され，公共用水域への年間排出量15t以上で，水より蒸気圧が低い有機化学物質群の中から20種類の化学物質（**Table 1**）を被検物質として選定した。直鎖ドデシルベンゼンスルホン酸ナトリウム（linear dodecyl-benzenesulfonate；DBS）は，直鎖アルキルベンゼンスルホン酸塩（linear-alkylbenzenesulfonate；LAS）の代表として選出した。また，ノニルフェノールジエトキシレート（nonylphenol diethoxylate；NP2EO）は，ノニルフェノールポリエトキシレート（nonylphenol polyethoxylate；

Table 1　Binding affinity of 20 PRTR chemicals for various nuclear receptors [a]

No.	Compound	ERα	TRα	RARγ	RXRα	VDR	PPARα	PPARγ	PPARδ
1	Ethylene glycol	–	–	–	–	–	–	–	–
2	N,N-Dimethylformamide	–	–	–	–	–	–	–	–
3	Nonylphenol diethoxylate (NP2EO)	++	–	–	–	–	–	+	–
4	Thiourea	–	–	–	–	–	–	–	–
5	ε-Caprolactam	–	–	–	–	–	–	–	–
6	Ethylenediaminetetraacetic acid	–	–	–	–	–	–	–	–
7	Terephthalic acid (TPA)	–	–	++	–	++	–	+	–
8	Diethylenetriamine	–	–	–	–	–	–	–	–
9	Toluene	–	–	–	–	–	–	–	–
10	1,4-Dioxane	–	–	–	–	–	–	–	–
11	Methacrylic acid	–	–	–	–	–	–	–	–
12	2-Aminoethanol	–	–	–	–	–	–	–	–
13	Acrylic acid	–	–	–	–	–	–	–	–
14	Linear dodecyl-benzenesulfonate (DBS)	–	+	++	–	++	–	+	–
15	1,3-Dichloro-2-propanol	–	–	–	–	–	–	–	–
16	Hexamethylenediamine	–	–	–	–	–	–	–	–
17	*p*-Xylene	–	–	–	–	–	–	–	–
18	Aniline	–	–	–	–	–	–	–	–
19	Pyridine	–	–	–	–	–	–	–	–
20	Phenol	–	–	–	–	–	–	–	–

[a] ++, the lowest detectable effective concentrations of tested chemicals were 10^3 to 10^5 times as much as that of the cognate ligand; +, 10^6 to 10^8 times; –, not detected.

NPnEOs）の代表として使用した。NP2EOは，親物質であるNPnEOsにも含まれているが，NPnEOsの水環境中での分解の最終産物の一つである。

エチレングリコール，チオ尿素，トルエン，2-アミノエタノール，DBS，1,3-ジクロロ-2-プロパノール，*p*-キシレンは和光純薬工業，N,N-ジメチルホルムアミド，ε-カプロラクタム，テレフタル酸（telephthalic acid；TPA），1,4-ジオキサン，アニリン，フェノールはキシダ化学，NP2EO，エチレンジアミン四酢酸，ジエチレントリアミン，メタクリル酸，アクリル酸，ヘキサメチレンジアミンは東京化成工業，ピリジンは林純薬工業から購入した。

標準リガンド及び被検化学物質は，DMSOに溶解後4 ℃で保存し，使用前にDMSOで段階希釈して用いた。

2.2 CoA-BAP法

本研究では，西川らが開発したCoA-BAP法[18]を用いて，供試化学物質のNRへの結合を評価した。CoA-BAP法は，生細胞を使用せずに，リガンドに依存したNRとコアクチベーターの相互作用をマイクロプレート上で検出する*in vitro*手法である。本手法では，大腸菌を用いて予め高発現させたNRリガンド結合領域（NR-LBD）とコアクチベーター（CoA）を使用する。NR-LBDを固定したプレートにCoAとリガンドを加えると，リガンドに依存してNRの立体構造が変化し，CoAがNR-LBDに結合する。CoAにはBAPが融合されているため，NRとCoAの相互作用の強さは，BAPのアルカリフォスファターゼ（AP）活性の強さとして測定できる。このAP活性は用量反応性を示し，検出感度が酵母two-hybrid法よりも高いことが確認されている[18]。また本法は，マイクロプレート上で操作するため，一度に多数の化学物質を試験することができる。さらに，NR-LBDとCoAをタンパクとして用いているため，これまで環境汚染化学物質のホルモン様活性の検出に汎用されてきた酵母法で問題視されてきた酵母細胞膜への透過性や酵母細胞に対する毒性による影響を回避することが可能である。

CoA-BAP法は，上述した原理に則って作製されたNuLigandシリーズ（マイクロシステムズ）を用いて行った。0.1 M炭酸緩衝液に溶解させた受容体を96穴マイクロプレートに分注し，4 ℃で一晩静置することでウェルに固定化させた。緩衝液A（Tris-HCl 20 mM, KCl 100 mM, EDTA 0.25 mM, glycerol 5%, dithiothreitol 0.5 mM, Tween 20 0.05%, pH 7.2）でウェルを3回洗浄後，緩衝液Aに懸濁させた30 $\mu g \cdot ml^{-1}$のTIF2-BAP（PPARδ以外）或いはCBP-BAP（PPARδ）を100 μl分注し，適宜希釈した標準リガンド或いは被検物質を添加して，4 ℃で1時間静置した。緩衝液B（Tris-HCl 50 mM, KCl 100 mM, $MgCl_2$ 5 mM, Nonidet P-40 0.1%, pH 7.2）でウェルを3回洗浄後，発色基質（*p*-nitrophenylphosphoric acid 10 mM, Tris-HCl 100 mM, pH 8.0）を100 μl添加して，30 ℃或いは37 ℃で反応させ，405 nmの吸光度（A_{405}）を測定した。実験は全て3連で行い，平均と標準偏差（standard deviation；SD）を算出した。ある被検物質濃度におけるA_{405}値の平均- SDがDMSOのA_{405}値の平均+SDを上回り，それより高濃度でA_{405}値がさらに上昇した場合に陽性と判定した。

3．結　　果

CoA-BAP法により，10^{-9}-10^{-3} Mの範囲で，20種のPRTR化学物質の各種NRに対する結合性を調べた。17種類の化合物では，いずれのNRに対しても有意な結合性を示さなかったが，NP2EO，TPA，DBSの3物質はそれぞれ2，3，4種類のNRに結合した（**Table 1**）。NRで見ると，RXRα，PPARα，PPARδを除く5種類のNRに対して，少なくとも1種類の被検化学物質が結合した。活性を示した物質の用量反応曲線を受容体種ごとに**Fig. 1**に示す。

NP2EOは，ERαとPPARγに対して結合性を示した。ERαでは，標準リガンドであるE2に比べて10^3倍高濃度である10^{-6} Mから結合性が確認された（**Fig. 1A**）。一方，PPARγへの結合性が認められる濃度は標準リガンドに比べて10^6倍高濃度であり，活性は非常に低かった（**Fig. 1E**）。

TPAは，RARγ，VDR及びPPARγに対して結合性を示した。RARγに対する結合は10^{-7} Mから認められ，それより高濃度では濃度依存的に活性が上昇した（**Fig. 1C**）。VDRに対しては，10^{-8} Mで活性を示し，それより高濃度では，$1,25(OH)_2D3$と同様の割合で，濃度依存的に活性が上昇した（**Fig. 1D**）。PPARγにおいては，10^{-6} M以上の濃度で結合したが，それより高濃度における活性の増大は緩やかであった（**Fig. 1E**）。

DBSは，TRα，RARγ，VDR，PPARγの4種類のNRに結合した。TRαに対する結合性は，10^{-4} M以上の濃度でのみ認められた（**Fig. 1B**）。PPARγに対しては，10^{-6} Mで結合性を示した。その活性は10^{-6}-10^{-4} Mにおいて僅かずつ高まったが，10^{-4} M以上では変化しなかった。RARγとVDRに対しては，それぞれ10^{-6} M及び10^{-7} Mで結合性を示した。また，これらより高濃度では，それぞれの標準リガンドの用量反応曲線に類似した割合で濃度依存的に活性が増大した（**Fig. 1C, D**）。

4．考　　察

従来，内分泌攪乱活性に基づく環境汚染化学物質のリスク評価は，主にERとTRを対象として進められ，現在EDsとして疑いのある物質に挙げられているアルキルフェノール類やフタル酸エステル類，ビスフェノールA，農薬類などに対して数多くの知見が得られてきた。本研究で調べた20種類のPRTR化学物質の中で，NP2EOがERαに結合することが観察された。NP2EOは，酵母法[19]やマスのビテロゲニン遺伝子発現試験[20]においてエストロゲン様活性が確認されている。また，本研究で得られたNP2EOのERαに対する最少活性発現濃度は，既往研究[18]における4-ノニルフェノールのERαに対する最少活性発現濃度の1/100-1/10であり，これは酵母法における結果[19]と同等である。これらより，CoA-BAP法で得られたNRに対する結合性はある程度妥当なものであると考えられた。他方，TRαには，10^{-4} M以上の高濃度でDBSが結合する可能性のあることが示されたが，DBSを含むLASの甲状腺ホルモン様活性に関する報告はこれまでにない。

これまで，性ホルモン受容体とTR以外のNRは水環境中における化学物質のリスク評価に考慮されてこなかった。そこで本研究では，新たな試みとして，公共用水域に大量に排出されている化学物質を対象として，ER，TR以外のNRへの結合性を調べた。その結果，NP2EOがPPARγ，TPAがRARγ，VDR及びPPARγ，DBSがRARγ，VDR及びPPARγに結合する可能性のあることが確認された。特に，TPAとDBSのRARγ及びVDRに対する結合性は標準リガン

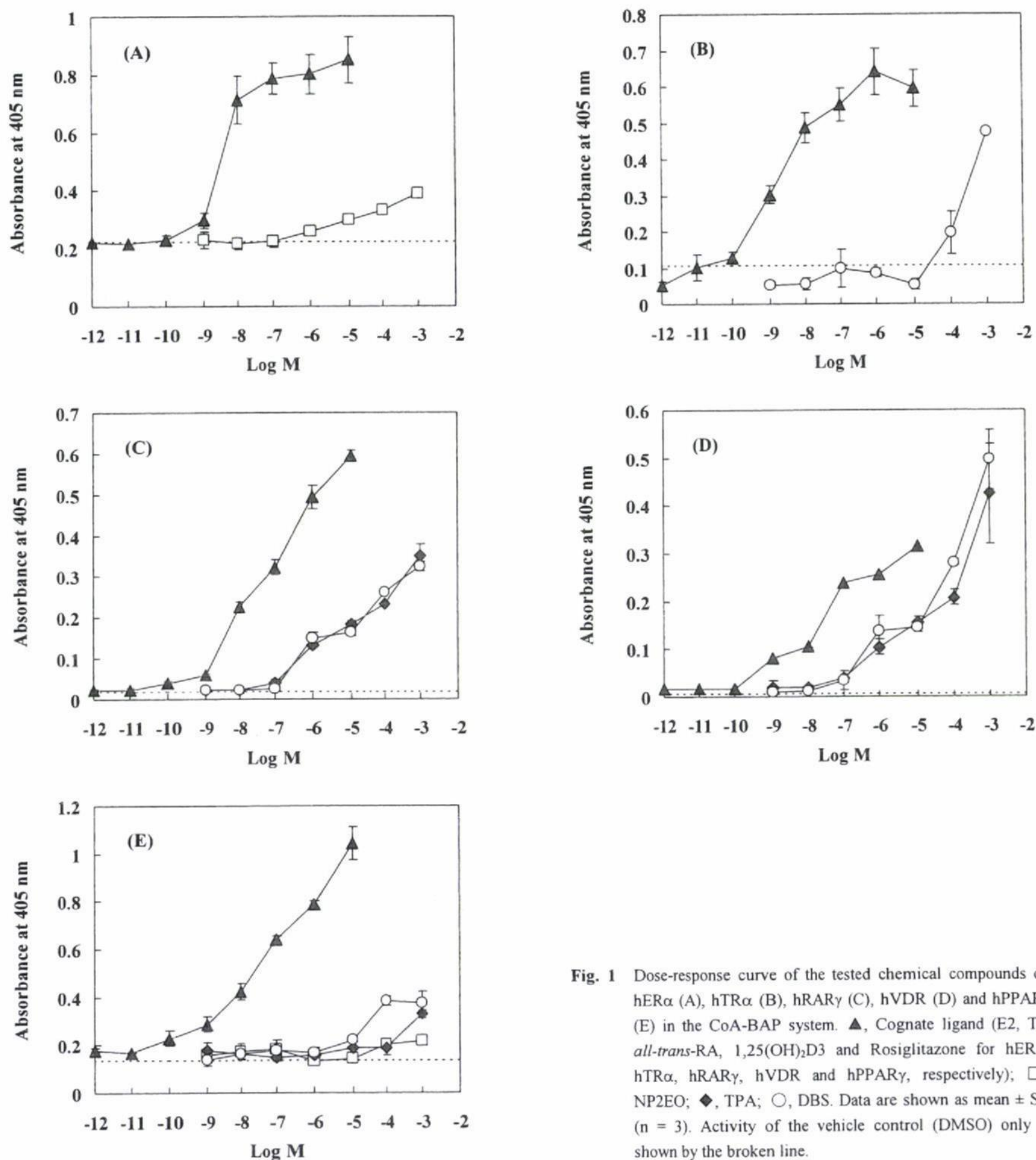

Fig. 1 Dose-response curve of the tested chemical compounds on hERα (A), hTRα (B), hRARγ (C), hVDR (D) and hPPARγ (E) in the CoA-BAP system. ▲, Cognate ligand (E2, T3, *all-trans*-RA, 1,25(OH)$_2$D3 and Rosiglitazone for hERα, hTRα, hRARγ, hVDR and hPPARγ, respectively); □, NP2EO; ◆, TPA; ○, DBS. Data are shown as mean ± SD (n = 3). Activity of the vehicle control (DMSO) only is shown by the broken line.

ドに類似した割合で濃度依存的に増大した(**Fig. 1C, D**)。これらの結果は，現在使用されている多種多様な化学物質の中に，48種類存在するNRのいずれか或いは複数に作用して，内分泌撹乱活性を示すものが数多く存在していることを強く示唆するものである。このため，今後のEDsの評価では，種々のNRを介した内分泌撹乱活性のスクリーニングを実施していくことが重要である。

本研究は被検化学物質のNRに対する結合性をスクリーニングしたものであり，陽性と判定されたケースで必ずしも生体影響が生じる訳ではない。しかし，本研究の結果はEDsによる内分泌撹乱作用の第一段階であるNRとの結合が生じる可能性を示すものであり，被検化学物質の潜在的な内分泌撹乱作用の可能性をある程度推測し得るものと言える。また，NRには種差があるため，ヒトNRを用いた本研究の結果が全野生生物種に当てはまる訳ではない。しかし，NRの種類によっては，異種間で高い保存性を示すものもあることから[9]，本研究の結果に基づき，ヒト以外の野生生物種を含め，被検物質の生物への悪影

響の可能性をある程度推察できるものと考えられる。そこで，以下では，本研究で得られた結果を基に，幾つかのNRに対する結合性が確認されたNP2EO，TPA及びDBSの潜在的な内分泌攪乱作用に関する推察を試みた。

RARは，脊椎動物の視覚や形態形成，発生，細胞分化，組織の恒常性に重要な役割を果たしている。RARのリガンドであるRAは，脊椎動物の催奇形物質であり，その過剰摂取は多種多様な奇形を発生させる[21,22]。1990年代から北米で観察されているカエルの奇形にも，水環境汚染化学物質によるRARシグナル伝達系の攪乱が関係していることが指摘されている[23,24]。VDRは，カルシウムの恒常性，骨代謝，及び他の重要な生物作用（細胞分化の誘導，細胞増殖阻害，免疫修飾，他のホルモン系の制御など）において中心的な役割を担っている[25]。PPARγは，主に脂肪とグルコースの代謝に重要な役割を果たしており，種々の臓器において抗発癌作用を発現する[26]。また，そのアゴニストは，臨床でII型糖尿病の治療薬として使用されている。現在のところ，環境中でVDR或いはPPARγを介したものと考えられる悪影響の観察事例はない。

RARγ[27]とPPARγ[26]は，RXRとヘテロ二量体を形成し，各々の標的遺伝子を転写活性化する。しかし，これらの受容体への結合性が認められたNP2EO（PPARγ），TPA（RARγ, PPARγ），DBS（RARγ, PPARγ）は，いずれもRXRαには結合しない（**Table 1**）。NRが二量体を形成して活性化されるシグナル経路においては，リガンドが二量体を成すNRの片方にしか結合しない場合の影響は，双方に結合する場合に比べて小さい可能性が指摘されている[9]。このことから，NP2EO，TPA，DBSが単独でRARやPPARγを介するシグナル伝達系を攪乱する可能性は小さいことが示唆された。しかし，有機スズ化合物のようにRXRαに結合する化学物質が共存する環境下では，RARやPPARγのシグナル伝達系への悪影響が生じる可能性もあり得る。ただし，RARとPPARγに対する最少活性発現濃度が環境水中濃度に比べて高いことから（NP2EOで10^4倍以上[28]，TPAで100倍以上[29]，DBSで5倍以上[30]），現状ではこれらの物質が生物に悪影響を及ぼしている可能性は低いものと考えられる。

他方，VDRは，ホモ二量体，或いはRARかRXRとのヘテロ二量体を形成し，VD応答遺伝子を転写制御する[31,32]。VDRへの結合が確認されたTPAとDBSはRXRに結合しないことから，上述したRAR及びPPARγのケースと同様に，VDR-RXRヘテロダイマーを介するVDRシグナル伝達機能への悪影響の可能性は低いものと予想される。一方，TPAとDBSは，RARγとVDRの両受容体に対する結合性が濃度依存的に増大したことから，それぞれ単独でVDRホモ二量体或いはVDR-RARヘテロ二量体に強く作用する可能性がある。TPAは，混餌投与によって，Fischer-344ラット離乳児に対して，TPAカルシウムを主成分とする膀胱結石を形成し，膀胱移行上皮の肥厚化と高カルシウム尿症を引き起こすことが報告されている[33]。また，DBSを含むLASは，経口投与によって哺乳動物胎児に骨化遅延を引き起こすことが明らかにされている[34]。すなわち，両物質が有する生体毒性は，VDRシグナル伝達系が関わる生体内機能と興味深い一致を示している。以上のことから，TPAとDBSは，急性毒性を示さない濃度において，VDRホモ二量体或いはVDR-RARヘテロ二量体に作用することでVDRシグナル伝達機能を乱し，生体に悪影響を及ぼす可能性のあることが示唆された。しかし，TPAは，環境水中濃度がRARγ及びVDRへの最少活性発現濃度よりもそれぞれ100倍及び10倍以上低く，また，生分解性が良好で残留性が低いことから[29]，実環境中で活性が発現する可能性は高くないものと推察される。他方，DBSは，環境水中濃度がRARγ及びVDRに対する最少活性発現濃度と同程度の場合もあることから[30]，活性発現の可能性を完全には否定できないが，水環境中での生分解性が高く，数時間～数日間で消失することから[30]，リスクはさほど高くないものと推定される。以上のように，現状のデータからでは，TPAやDBSが単独で野生動物のVDRシグナル伝達機能に悪影響を及ぼす可能性は低いと考えられるものの，これらが共存した場合には，相乗的な作用が生じることも否定できない。また，環境中への排出実態が明らかでない微量化学物質の中にもNRに結合性を示すものが存在すると推測されることから，多様なNRへの結合を介したリスクについて評価していくことが望まれる。

5．まとめ

本研究では，公共用水域への排出量の多いPRTR第1種指定化学物質20種のNR結合性を調査した。その結果，これまでに内分泌攪乱活性が疑われてこなかったTPAとDBSが複数のNRに結合する可能性のあることが示された。このことは，現在の評価体系で断定されている，或いは疑われているEDs以外の物質が，性ホルモン受容体やTR以外のNRを通じて，内分泌攪乱作用を示す可能性のあることを強く示唆している。今後の内分泌攪乱作用に関するリスク評価では，環境中に排出される可能性のある多様な人工化学物質を対象とし，種々のNRへの結合を介したリスクについて，網羅的，総合的に評価していくことが重要である。

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AIB1 Promotes DNA Replication by JNK Repression and AKT Activation during Cellular Stress

Kikumi Horiguchi, Shigeki Arai*, Tsutomu Nishihara and Jun-ichi Nishikawa†
Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka, 565-0871

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Amplified in breast cancer 1 (AIB1) is a member of the p160 family of nuclear receptor coactivator protein. Recent studies have reported that high-level AIB1 production is involved in the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway for progression to malignant carcinoma in a steroid-independent manner. Here we demonstrate that, in AIB1-knockout DT40 chicken B-lymphocytes, loss of AIB1 results in induction of phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun, in addition to the inhibition of DNA replication. In contrast, high-level AIB1 production prevents proapoptotic activation of the JNK/c-Jun signal transduction pathway and induces DNA replication through phosphorylation of the Akt/p65 NF-κB subunit RelA under cellular stresses such as UV irradiation or serum deprivation. Moreover, we have found that AIB1 is essential for the phosphorylation of histone H3 at serine 10, which is associated with the signal transduction to chromatin, leading to the transient expression of immediate-early genes in response to UV stimulation. Our results therefore suggest that AIB1 directly links to cell cycle control mechanisms in concern with the balance between apoptosis and proliferation.

Key words: amplified in breast cancer 1, cellular stress, DNA replication, phosphorylation, signal transduction.

Abbreviations: AIB1, amplified in breast cancer 1; Akt, cellular homolog of v-akt oncogene; CARM1, coactivator-associated arginine methyltransferase 1; CBP, cyclic AMP response element binding protein; CDK, cyclin dependent kinase; ER, estrogen receptor; ERK, extracellular signal–regulated kinase; FCS, fetal cauf serum; FACS, fluorence-activated cell sorting; GSK3, glycogen synthase kinase 3; HAT, histone acetyltransferase; HER-2, human epidemal growth factor receptor-2; JNK, c-Jun amino-terminal kinase; MAPK, mitosis-activated protein kinase; NF-κB, nuclear factor-κB; PAS, Per/Arnt/Sim; PI3K, phosphatidylinositol 3 kinase; RSK2, ribosomal S6 kinase 2; SRC-1, steroid receptor coactivator-1; TIF2, transcriptional intermediary factor 2; TUNEL, terminal deoxynucleotidyl transferase–mediated nick end label.

The nuclear receptor coactivator known as AIB1 (also called p/CIP, ACTR, RAC3 and SRC-3) is a member of the p160 nuclear receptor coactivator family. This family contains SRC-1 (steroid receptor coactivator-1) and TIF2 (transcriptional intermediate factor-2) that interact with the general transcriptional coactivators CBP, p300 and p/CAF (*1–8*). These coactivator complexes possess intrinsic histone acetyl transferase activity and are responsible for the remodelling of chromatin and modification of components of the transcription machinery (*9, 10*).

AIB1 increases estrogen-dependent transcriptional activation by interaction with estrogen receptor (ER) α in a ligand-dependent manner. Furthermore, AIB1 mRNA and protein have been shown to be amplified and overexpressed in primary human breast and ovarian cancer cell lines, in which transcription is upregulated and the AIB1 gene on chromosome 20q12 is amplified (*1*). Recent studies report that high levels of AIB1 production are relates to both a high DNA-synthesis phase fraction and HER-2/*neu* production with p53 mutations in breast cancer, which is a disease characterized by an imbalance between cell division and cell death (*11, 12*). Her-2/*neu* protein activates the PI3K (phosphoinositide 3-kinase)/Akt (also known as protein kinase B, PKB) pathway, which, through NF-κB activation, plays an important role in preventing cells from undergoing apoptosis (*13, 14*). More recently, it has been shown that overexpression of AIB1 in the mammary gland leads to activation of the PI3K/Akt pathway, with IGF-1 signaling (*15*). Because AIB1 (RAC-3) has also been shown to interact with NF-κB and enhance its transcriptional activity (*16, 17*), it has been suggested that AIB1 is an altered regulator for the mechanism by which constitutive activity of an NF-κB–dependent promoter is involved in chemotherapeutic resistance in ER-negative cancer cells (*18, 19*). However, the biological function of AIB1 in the signal transduction pathways influenced by complex cascades of phosphorylation events triggered by exposure to cellular stress is not completely understood. Therefore, we focused our attention on AIB1 activity, to determine whether this protein regulates the antiapoptotic process or perturbs signal integration in response to cellular stress.

Importantly, c-Jun N-terminal kinase (JNK) has also been shown to be a key regulator of programmed cell death and part of a subfamily of the mitogen-activated protein kinase (MAPK) superfamily (*20*). Recent studies indicate that JNK activation contributes to

*Present address: Research Center for Genomic Medicine, Saitama Medical School, 1397-1 Yamane, Hidaka, Saitama 350-1241.
†To whom correspondence should be addressed. Tel: +81-6-6879-8241, Fax: +81-6-6879-8244, E-mail: nisikawa@phs.osaka-u.ac.jp

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