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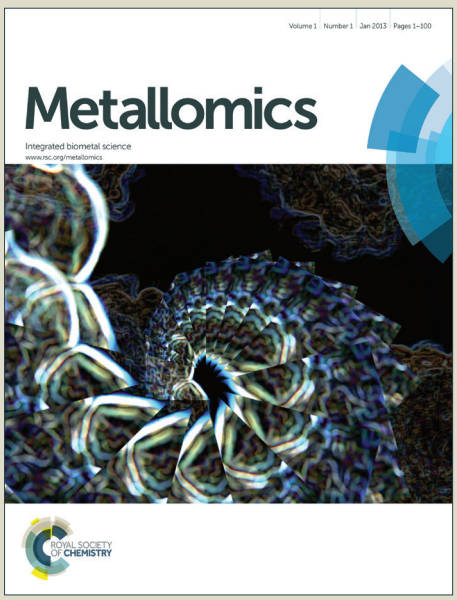

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Transactivation of human retinoid X receptor by organotins: Use of site-directed mutagenesis to identify critical amino acid residues for organotin-induced transactivation

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Abstract

Organotins, such as tributyltin (TBT) and triphenyltin (TPT), may disrupt endocrine activity in mammals arising from their ability to act as ligands for the retinoid X receptor (RXR) and peroxisome proliferator-activated receptor γ (PPARγ). The structure of TBT is completely different from that of 9-*cis* retinoic acid (9cRA), an endogenous RXR ligand; and X-ray crystallographic studies have revealed that TBT and 9cRA have distinct binding interactions with human RXRα. Therefore, organotins and rexinoids likely activate RXR by different mechanisms. Here, we used human RXRα mutants to investigate which amino acid residues of the receptor are critical for transactivation induced by rexinoids and organotins. We found that 9cRA and a synthetic RXR agonist (LG100268) failed to activate R316A and L326A RXRα mutants. In contrast, all the tested organotins activated the R316A mutant, the L326A mutant, or both but failed to activate a C432A mutant. These results suggest that the importance of L326, which is located in the β-strand, for rexinoid-induced transactivation of RXRα is comparable to that of R316; in contrast, C432 is critical for organotin-induced transactivation, whereas R316 and L326 are not required. We used a PPARγ/RXRα C432A heterodimer to determine whether TBT and TPT could activate the heterodimer by binding to PPARγ. We found that TBT and TPT activated the PPARγ/RXRα C432A heterodimer, which suggests that both compounds can activate the heterodimer through PPARγ. These findings indicate that the amino acid residues that are critical for organotin-induced transactivation of RXRα are distinct from those required for rexinoid-induced transactivation.

Introduction

Organotins, such as tributyltin (TBT) and triphenyltin (TPT), have been widely used as antifouling biocides for ships and fishing nets. These compounds are known to interfere with sexual development and reproduction; for example, TBT and TPT show endocrine-disrupting activity in some species of gastropods, leading to the development of imposex [1, 2], that is, the superimposition of male genitalia on female genitalia. In addition, these compounds markedly enhance estradiol biosynthesis in human choriocarcinoma cells [3, 4] and are therefore suspected of also having endocrine-disrupting effects in mammals, including humans. Our previous work indicates that a critical event for endocrine disruption by organotins is transactivation of the retinoid X receptor (RXR) [5–7], a nuclear receptor superfamily member that specifically binds the naturally occurring rexinoid 9-*cis*-retinoic acid (9cRA) and thus may be directly involved in the transduction of rexinoid signals. RXR agonists can activate the RXR homodimer as well as heterodimers formed with permissive partners such as the peroxisome proliferator-activated receptor (PPAR), the liver X receptor, and the farnesoid X receptor [8–11]. Recently, we determined that TBT and TPT are nanomolar agonists for RXR [5, 6] and that other organotin compounds also have RXR agonist activity at concentrations that depend on both the number and the length of their alkyl chains [5, 7]. Furthermore, we found that organotin compounds induce imposex in gastropods and modify the endocrine function of the human placenta *via* the RXR signaling pathway [5, 12]. However, organotin compounds do not resemble known rexinoids either structurally or chemically. Rexinoids contain a carboxylate group and a long aliphatic chain, and the crystal structure of the complex between human RXRα (hRXRα) and bound 9cRA shows that the carboxylate group interacts with Q275 in

helix 3, R316 in helix 5 and L326 in the β-strand and that the long aliphatic chain interacts with a subset of the binding-pocket residues in helix 11 [13, 14]. Organotin compounds, in contrast, lack a polar functional group, and therefore it is thought that the critical amino acid residues involved in organotin-induced transactivation of RXR differ from those involved in transactivation by well-known rexinoids. In addition, le Maire *et al.* recently solved the crystal structure of the hRXRα ligand-binding domain bound to TBT and reported that TBT occupies only a small part of the ligand-binding pocket and interacts with only a subset of the binding-pocket residues, including C432 in helix 11. In addition, these investigators found that mutation of C437 in mouse RXRα, which is equivalent to C432 in hRXRα, abrogates TBT-induced RXRα activity [15]. No such site-directed mutagenesis experiments have ever been done with hRXRα. We reported previously that TBT and TPT are also nanomolar agonists for PPARγ [6, 7], which is activated by a variety of fatty acids as well as thiazolidinedione antidiabetic agents, such as rosiglitazone [16]. PPARγ and RXR form a permissive heterodimer; that is, the heterodimer can be activated by both RXR and PPARγ agonists. Therefore, TBT and TPT may transactivate the RXR/PPARγ heterodimer by acting as RXR or PPARγ agonists. However, le Maire *et al.* suggested that TBT binds specifically to RXR rather than to PPARγ, owing to the absence of a suitable cysteine residue in the latter [15]. However, we recently demonstrated, using X-ray crystallography, mass spectroscopy, and a cell-based reporter assay, that TBT is anchored to C285 of human PPARγ by an ionic bond [17]. Therefore, whether the RXR/PPARγ heterodimer can be activated by binding of TBT or TPT to PPARγ remains an open question. In this study, to extend our knowledge of the mechanisms of RXR transactivation by organotin compounds, we used site-directed mutagenesis to investigate some hRXRα amino acid residues that

have the potential to be critical for rexinoid- and organotin-induced transactivation. Furthermore, we investigated whether RXR could be activated by binding of TBT or TPT to PPARγ.

Materials and methods

Chemicals

Tributyltin chloride (TBTCl) and triphenyltin hydroxide (TPTOH) were obtained from Tokyo Kasei Kougyo (Tokyo, Japan). Tricyclohexyltin hydroxide (TChTOH) and tetrabutyltin (TeBT) were obtained from Aldrich Chemicals (Milwaukee, WI). Tripropyltin chloride (TPrTCl) was obtained from Merck (Darmstadt, Germany). 9cRA was obtained from Sigma Chemical Co. (St. Louis, MO). LG100268 (LG) was obtained from Toronto Research Chemicals (North York, Ontario, Canada). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI). All chemicals were dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan).

Cell cultures

Human choriocarcinoma cells (JEG-3 cell line, ATCC HTB-36) were obtained from ATCC (Manassas, VA). JEG-3 cells were cultured in minimal essential medium containing 2 mM L-glutamine, 0.1 mM minimal essential medium nonessential amino acid solution (Invitrogen, Carlsbad, CA), and 10% fetal calf serum. For determination of the effects of organotin compounds on reporter gene expression, the cells were seeded on culture plates and precultured for 24 h and then treated with either organotin compounds or rexinoids in 0.1% DMSO or vehicle (0.1% DMSO) alone.

Plasmid construction

For the chimeric receptor assay, we used a pBK-CMV-GAL4-hRXRα plasmid, which was previously generated in our laboratory and consists of the ligand-binding domain (amino acids 201–462) of hRXRα fused to the C-terminal end of the GAL4 DNA-binding domain (amino acids 1–147) in the pBK-CMV expression vector (Stratagene, La Jolla, CA) [5]. We also used the PPARγ expression vector pSVhPPARγ, which was previously generated in our laboratory and consists of the full-length cDNA of human PPARγ in the pSVSPORT1 vector (Invitrogen) [7].

GAL4-hRXRα mutant constructs in which Q275, R316, L326, both R316 and L326, or C432 and an hPPARγ mutant construct in which C285 was mutated to alanine were generated by site-directed mutagenesis of the pBK-CMV-GAL4-hRXRα plasmid and the pSVhPPARγ plasmid, respectively, by using a PrimeSTAR Mutagenesis Basal Kit (Takara Bio, Shiga, Japan). The primers used to generate these mutants and the PCR conditions are shown in Table 1. The resulting mutant constructs are designated pBK-CMV-GAL4-hRXRαQ275A, pBK-CMV-GAL4-hRXRαR316A, pBK-CMV-GAL4-hRXRαL326A, pBK-CMV-GAL4-hRXRαR316A/L326A, pBK-CMV-GAL4-hRXRαC432A, and pSV-PPARγC285A, respectively. All sequences synthesized by PCR were confirmed by DNA sequencing. The luciferase (LUC) reporter construct containing 4 copies of the GAL4 DNA-binding site (upstream activation sequence; UAS) followed by the thymidine kinase promoter (p4×UAS-tk-luc) used in the chimeric receptor assay were kindly provided by Dr. Y. Kamei (National Institute of Health and Nutrition, Japan).

Transient transfection assay

Transient transfection assay was performed in accordance with our previous study [17]. Briefly, JEG-3 cells (3×10^4 cells) were seeded in 24-well plates 24 h before transfection with the optimal doses of each DNA construct. At 24 h after transfection, various test compounds were added to the transfected cells, which were then cultured in regular culture medium supplemented with 1% charcoal-stripped fetal calf serum instead of 10% normal fetal calf serum. The cells were harvested 24 h later, and extracts were prepared and assayed for firefly LUC activity. For normalization of firefly LUC activity for transfection and harvesting efficiency, the *Renilla* LUC control reporter construct pGL 4.74 (Promega) was cotransfected as an internal standard in all reporter experiments. The results are expressed as the average relative firefly LUC activity of at least quadruplicate samples.

Statistic analysis

All data from the control and treatment groups were obtained from the same numbers of replicated experiments. In addition, all the experiments were carried out independently 2 or 3 times. Tukey's multiple comparisons test and 2-way analysis of variance were applied to the raw data using SPSS 15.0J software (SPSS, Chicago, IL). $P < 0.05$ was taken to indicate statistical significance.

Results

1. Effects of Q275A, R316A, and L326A mutations on hRXRα transcriptional responsiveness to 9cRA and TBTCl

The carboxylate group of 9cRA is reported to interact with R316 in helix 5 of hRXRα [13, 14]. In the current study, we prepared an R316A mutant, as well as mutants with alanine at Q275 in helix 3 and L326 in the β-sheet, and we evaluated the effects of these mutations on hRXRα transactivation induced by rexinoids and organotin compounds. First, we used a chimeric receptor assay to evaluate the ability of 9cRA and TBTCl to activate the Q275A, R316A, and L326A hRXRα mutants (Fig. 1), and we found that the Q275A mutant was activated by both 9cRA and TBTCl to an extent comparable to that of the wild-type RXRα. In contrast, although the activity of TBTCl with respect to the R316A and L326A mutants was almost the same as that with respect to the wild type, the responsiveness of the R316A and L326A mutants to 9cRA was entirely lost.

2. Effects of R316A mutation on transcriptional responsiveness of hRXRα to rexinoids and organotin compounds

To further investigate the functional potency of R316 in hRXRα transactivation induced by rexinoids and organotin compounds, we investigated the ability of rexinoids and organotin compounds to activate the R316A hRXRα mutant. Consistent with our previous observations [5], 100 nM and 1 μM 9cRA; 1, 10, and 100 nM LG (a synthetic RXR agonist); 10, 30, and 100 nM TBTCl; 10, 30, and 100 nM TPTOH; 10, 30, and 100 nM TPrTCl; 30 and 100 nM TChTOH; and 1, 3, and 10 μM TeBT induced transactivation of wild-type hRXRα (Figs. 2 and 3). In contrast, the concentration of

9cRA required to elicit a response from the R316A mutant was about 1 order of magnitude higher than that required for the wild type (Fig. 2). In addition, the maximum fold-activation value was also lower for the mutant than for the wild-type, indicating that the response of the former to 9cRA was significantly reduced. In addition, the mutant exhibited significantly lower responsiveness to LG relative to that of the wild type (Fig. 2). The concentrations of TBTCl and TChTOH required to elicit a response from the R316A mutant were almost equivalent to those required for the wild-type, but the maximum fold-activation values were significantly lower for the mutants (Figs. 2 and 3). The R316A mutant showed almost the same response to TPTOH and TPrTCl as the wild-type (Figs. 2 and 3). The concentration of TeBT required to elicit a response from the R316A mutant was about 3 times that required for the wild-type, and the maximum fold-activation value was significantly lower for the mutant (Fig. 3).

3. Effects of C432A mutation on transcriptional responsiveness of hRXRα to rexinoids and organotin compounds

According to our previous work [16] and that of others [14], the tin atom of TBT forms an ionic bond with the sulfur atom of C432 of hRXRα, but the activity of organotin compounds with respect to a C432A-mutant hRXRα has not previously been investigated. To determine the functional potency of C432 with respect to hRXRα transactivation induced by rexinoids and organotin compounds, we investigated the ability of rexinoids and organotin compounds to activate a C432A mutant. Although the response of the mutant to 9cRA and LG was lower than the response of the wild type, the response was not entirely lost (Fig. 2). In contrast, none of the tested organotin compounds elicited any response from the C432A mutant (Figs. 2 and 3), suggesting

that C432 is critical for organotin-induced transactivation of hRXRα.

4. Effects of L326A mutation on transcriptional responsiveness of hRXRα to rexinoids and organotin compounds

We also investigated the ability of the rexinoids and organotin compounds to activate the L326A-mutant hRXRα because L326 was involved in the transactivation of hRXRα by 9cRA but not by TBTCl, as shown in Fig. 1. Like the R316A mutant, the L326A mutant was significantly less responsive to 9cRA and LG than the wild type. The L326A mutation significantly reduced the ability of hRXRα to be activated by TBTCl and TPTOH, but the concentrations of TBTCl and TPTOH required to elicit a response from the L326A mutant were about 1 order of magnitude higher than the concentrations required for the wild type (Fig. 4). The concentrations of TPrTCl and TeBT required to elicit a response from the L326A mutant were about 1 order of magnitude higher than the concentration required for the wild type, and the maximum fold-activation value was also lower for the mutant than for the wild type (Fig. 5). In contrast, the L326A mutant and the wild type showed almost the same responsiveness to TChTOH (Fig. 5).We also generated hRXRα with alanine mutations at both R316 and L326 (designated R316A/L326A) and investigated the ability of rexinoids, TBTCl, and TPTOH to activate this double mutant. The R316A/L326A mutant exhibited almost no responsiveness to either 9cRA or LG (Fig. 4). The double mutant was less responsive to TBTCl and TPTOH than the wild type and showed responsiveness similar to that of the L326A mutant (Fig. 4). The R316A/L326A mutant was also significantly less responsive to TPrTCl and TeBT than the wild type (Fig. 5). Nevertheless, the maximum fold-activation value of the R316A mutant resulting from treatment with TChTOH was

significantly reduced relative to that of the wild type, whereas the double mutant showed almost the same responsiveness to TChTOH as did the wild type (Fig. 5). However, the maximum fold-activation value resulting from treatment of the R316A/L326A mutant with TPrTCl or TeBT was slightly higher than that observed for the L326A mutant (Fig. 5).

5. Effect of hPPARγ–organotin complexes on transactivation of hRXRα

As mentioned above, previous work has not clarified whether RXR can be activated by PPARγ–organotin complexes. In this study, we found that organotin compounds completely failed to activate C432A-mutant hRXRα (Figs. 2 and 3). On the basis of these results, we investigated the ability of hPPARγ–TBTCl and hPPARγ–TPTOH complexes to activate hRXRα, by using JEG-3 cells transfected with pBK-CMV-GAL4-hRXRαC432A and with or without a hPPARγ expression vector. We expected that in the presence of both the hPPARγ expression vector and the GAL4-RXRαC432A expression vector, C432A-mutant hRXRα would form with hPPARγ, and if organotin compounds could function as PPARγ agonists, they would be able to activate C432A-mutant hRXRα *via* hPPARγ (Fig. 6). We found that in the absence of the hPPARγ expression vector, that is, in the presence of only the GAL4-hRXRαC432A expression vector, 9cRA increased luciferase activity relative to control, whereas TBTCl, TPTOH, and rosiglitazone (a typical synthetic PPARγ agonist) did not affect luciferase activity (Fig. 7A). In contrast, in the presence of the wild-type hPPARγ expression vector, that is, when GAL4-hRXRαC432A and wild-type hPPARγ formed a heterodimer, not only 9cRA and rosiglitazone but also TBTCl and TPTOH increased luciferase activity (Fig. 7B). However, when the expression vector of

C285A-mutant hPPARγ, a mutant that does not bind organotin [17], was used instead of the wild-type hPPARγ expression vector, TBTCl and TPTOH did not increase luciferase activity. (Fig. 7C).

Discussion

Previous studies of the hRXRα–9cRA complex showed that the carboxylate group interacts with R316 in helix 5 [13, 14] and that mutation of R316 reduces the binding affinity of hRXRα for 9cRA [18]. Unlike well-known rexinoids, organotins have no carboxylate group. Here, to identify some of the amino acid residues that are critical for hRXRα transactivation induced by rexinoids and organotin compounds, we investigated the ability of rexinoids and organotin compounds to activate various hRXRα mutants. The carboxylate group of rexinoids interacts not only with R316 but also with Q275 in helix 3 and L326 in the β-strand of hRXRα, as suggested by X-ray crystallographic studies [13, 14]. Although we found that Q275A mutation did not affect the ability of hRXRα to be activated by 9cRA or TBT (Fig. 1), L326A mutation significantly decreased the ability hRXRα to be activated by 9cRA and LG (Fig. 4). These results suggest that L326, but not Q275, is involved in rexinoid-induced transactivation of hRXRα.

The results of our experiments with the R316A and L326A hRXRα mutants suggested that even if an R316A and L326A double mutation abolished the ability of hRXRα to be activated by rexinoids, the double mutant would nevertheless still be activated by TBTCl and TPTOH. Indeed, we found that 9cRA completely failed to activate the double mutant; and although LG activated wild-type hRXRα at concentrations of 0.1 nM or more, LG failed to activate the R316A/L326A mutant at

concentrations below 100 nM (Figs. 2 and 4). In contrast, the R316A/L326A mutant was activated by TBTCl and TPTOH at 100 nM (Fig. 4). These results suggest that TBT and TPT bound specifically to the double mutant.

The maximum fold-activation value elicited by treatment of the R316A mutant with TBTCl or TeBT was significantly lower than that of the wild type (Figs. 2 and 3). In addition, the L326A mutation significantly reduced the ability of hRXRα to be activated by TeBT (Fig. 5). TBTCl also showed lower activity toward the L326A mutant, although the difference was not statistically significant (Fig. 4). These results suggest that the butyl substituent interacted with hRXRα *via* the R316 and L326 residues. By contrast, the activities of TPTOH and TPrTCl with respect to the R316A mutant were comparable to those with respect to the wild type (Figs. 2 and 3). However, the L326A mutation significantly reduced the ability of hRXRα to be activated by TPrTCl (Fig. 5). Although the L326A mutation did not result in significant reduction of hRXRα transactivation by TPTOH, the concentration required to elicit a response increased by approximately 1 order of magnitude (Fig. 4). These results indicate that L326 of hRXRα interacts with the propyl and phenyl substituents of the corresponding organotins.

Although the maximum fold-activation value resulting from treatment of the R316 mutant with TChTOH was significantly lower than that for the wild type (Fig. 3), TChTOH showed almost the same activity with respect to the L326A mutant as with respect to the wild type (Fig. 5). Furthermore, contrary to our expectation, TChTOH showed almost the same activity with respect to the R316A/L326A mutant and the wild type, suggesting that the decrement in TChTOH activity due to the R316A mutation was compensated for by the L326A mutation. Like the activity of TChTOH, the activities of

TPrTCl and TeBT with respect to the R316/L326A double mutant were slightly higher than those with respect to the L326A single mutant (Fig. 5). A conformational change in hRXRα induced by the R316A mutation might somehow have increased the susceptibility of the L326A mutant to these organotins.

RXR forms heterodimeric complexes with one-third of the 48 members of the human nuclear receptor superfamily [19], and the formation of these complexes enables the transactivation of multiple signaling pathways in both ligand-dependent and ligand-independent manners. RXR partners can be classified into functionally distinct permissive and nonpermissive groups [20]. In some heterodimers, such as RXR/thyroid hormone receptor and RXR/vitamin D receptor, RXR may be a completely silent partner. In others, such as the RXR/retinoic acid receptor, RXR seems to be a conditionally silent partner. In the PPAR/RXR heterodimer, RXR appears to be a fully active and competent partner [11, 21, 22], and RXR can therefore be activated by a PPARγ–agonist complex. However, le Maire *et al.* suggested that TBT binds specifically to RXR, rather than to PPARγ, because of the absence of a suitable cysteine residue in the latter [15]. Contrary to this suggestion, we recently demonstrated that TBT and TPT bind specifically to hPPARγ through an ionic interaction between the sulfur atom of C285 of hPPARγ and the tin atom [17]. Our previously reported data suggest that organotin compounds transactivate the PPARγ/RXR heterodimer by acting as PPARγ agonists. Accordingly, in the current study, we used C432A-mutant hRXRα to investigate the ability of hPPARγ–TBTCl and hPPARγ–TPTOH complexes to activate hRXRα. Predictably, the mutant hRXRα was activated by both TBTCl and TPTOH in the presence of wild-type hPPARγ, but not in the presence of C285A-mutant hPPARγ (Fig. 7). This result suggests that organotin compounds transactivate the PPARγ/RXR

heterodimer by acting as PPARγ agonists and that TBTCl and TPTOH may exert a synergistic effect by acting as agonists for both PPARγ and RXR.

In summary, we determined that C432 of helix H11 was critical for transactivation of hRXRα not only by TBT but also by other organotin compounds. The importance of L326 of the β-strand for rexinoid-induced transactivation of hRXRα was comparable to that of R316 of helix 5. R316 and L326 were also involved in the transactivation of RXR by some of the tested organotin compounds. In addition, we demonstrated that TBT and TPT could activate a PPARγ/RXR heterodimer through PPARγ. Our current observations may facilitate the design of new types of RXR agonists whose binding modes differ from the binding mode of known rexinoids.

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Figure legends

Fig. 1. Ability of 9cRA and TBTCl to activate Q275A, R316A, and L326A RXRα mutants. JEG-3 cells were cotransfected with 10 ng of p4×UAS-tk-luc and 5 ng of pBK-CMV-GAL4-hRXRα (wild type, Q275A, R316A, or C432A) by using Lipofectamine reagent and were then treated with 100 nM 9cRA or 100 nM TBTCl. To normalize luciferase activity for transfection and harvesting efficiency, the *Renilla* luciferase control reporter construct pGL4.74 was cotransfected as an internal standard in all reporter experiments. Results are expressed as average fold activation ± 1 SD of quadruplicate cultures.

Fig. 2. Ability of rexinoids, TBTCl, and TPTOH to activate R316A and C432A RXRα mutants. JEG-3 cells were cotransfected with 10 ng of p4×UAS-tk-luc and 5 ng of pBK-CMV-GAL4-hRXRα (wild type, R316A, or C432A) by using Lipofectamine reagent and were then treated with 9cRA, LG, TBTCl, TPTOH, or vehicle alone (control). To normalize luciferase activity for transfection and harvesting efficiency, the *Renilla* luciferase control reporter construct pGL4.74 was cotransfected as an internal standard in all reporter experiments. Results are expressed as average fold activation ± 1 SD of quadruplicate cultures. Lowercase "a" indicates a value significantly different from 0 nM ($P < 0.05$); * indicates a significant difference between 2 groups as determined by 2-way analysis of variance ($P < 0.05$).

Fig. 3. Ability of organotin compounds to activate R316A and C432A RXRα mutants. JEG-3 cells were cotransfected with 50 ng of p4×UAS-tk-luc and 20 ng of pBK-CMV-GAL4-hRXRα (wild type, R316A, or C432A) by using Lipofectamine

reagent and were then treated with TPrTCl, TChTOH, TeBT, or vehicle alone (control). To normalize luciferase activity for transfection and harvesting efficiency, the *Renilla* luciferase control reporter construct pGL4.74 was cotransfected as an internal standard in all reporter experiments. Results are expressed as average fold activation ± 1 SD of quadruplicate cultures. Lowercase "a" indicates a value significantly different from 0 nM ($P < 0.05$); * indicates a significant difference between 2 groups as determined by 2-way analysis of variance ($P < 0.05$).

Fig. 4. Ability of rexinoids, TBTCl, and TPTOH to activate L326A and R316A/L326A RXRα mutants. JEG-3 cells were cotransfected with 50 ng of p4×UAS-tk-luc and 20 ng of pBK-CMV-GAL4-hRXRα (wild type, L326A, or R316A/L326A) by using Lipofectamine reagent and were then treated with 9cRA, LG, TBTCl, TPTOH, or vehicle alone (control). To normalize luciferase activity for transfection and harvesting efficiency, the *Renilla* luciferase control reporter construct pGL4.74 was cotransfected as an internal standard in all reporter experiments. Results are expressed as average fold activation ± 1 SD of quadruplicate cultures. Lowercase "a" indicates a value significantly different from 0 nM ($P < 0.05$); * indicates a significant difference between 2 groups as determined by 2-way analysis of variance ($P < 0.05$). NS, not significant.

Fig. 5. Ability of organotin compounds to activate L326A and R316A/L326A RXRα mutants. JEG-3 cells were cotransfected with 50 ng of p4×UAS-tk-luc and 20 ng of pBK-CMV-GAL4-hRXRα (wild type, L326A, or R316A/L326A) by using Lipofectamine reagent and were then treated with TPrTCl, TChTOH, TeBT, or vehicle

alone (control). To normalize luciferase activity for transfection and harvesting efficiency, the *Renilla* luciferase control reporter construct pGL4.74 was cotransfected as an internal standard in all reporter experiments. Results are expressed as average fold activation ± 1 SD of quadruplicate cultures. Lowercase "a" indicates a value significantly different from 0 nM ($P < 0.05$); * indicates a significant difference between 2 groups as determined by 2-way analysis of variance ($P < 0.05$). NS, not significant.

Figure 6. The scheme of the investigation of the ability of hPPARγ–organotin complexes to transactivate hRXRα

Fig. 7. TBTCl and TPTOH activation of PPARγ/RXR heterodimer through PPARγ. JEG-3 cells were cotransfected with 20 ng of p4×UAS-tk-luc, 20 ng of pBK-CMV-GAL4-hRXRαC432A, and 20 ng of one of the following: pSV-SPORT (A), pSV-PPARγ (B), or pSV-PPARγC285A (C) and were then treated with 100 nM 9cRA, TBTCl, TPTOH, rosiglitazone (rosi), or vehicle alone (control). To normalize luciferase activity for transfection and harvesting efficiency, the *Renilla* luciferase control reporter construct pGL4.74 was cotransfected as an internal standard in all reporter experiments. Results are expressed as average fold activation ± 1 SD of quadruplicate cultures; * indicates a value significantly different from control ($P < 0.05$).

Table 1. Oligonucleotide primer sequences and PCR conditions for amino acid substitutions of pSV-PPARγ and pBK-CMV-GAL4-RXRα.

mutations	Primer sequences		PCR conditions			
		Sequence (5' to 3')	Denaturation	Annealing	Elongation	Cycle No.
RXRαQ275A	Forward	GAC AAA **GCA** CTT TTC ACC CTG GTG GA	98 °C	58 °C	72 °C	35
	Reverse	GAA AAG **TGC** TTT GTC GGC TGC TTG GCA A	15 s	5 s	30 s	
RXRαR316A	Forward	TTC TCC CAC **GCC** TCC ATC GCC GTG AA	98 °C	58 °C	72 °C	35
	Reverse	ATG GA**G GC**G TGG GAG AAG GAG GCG ATG A	15 s	5 s	30 s	
RXRαL326A, [a]R316A/L326A	Forward	TCC TC**G CA**G CCA CCG GGC TGC A	98 °C	58 °C	72 °C	35
	Reverse	CGG TGG C**TG C**GA GGA TCC CGT CCT TCA	15 s	5 s	30 s	
RXRαC432A	Forward	GGC TCA AA**G CA**C TGG AAC ATC TCTTCT	98 °C	58 °C	72 °C	35
	Reverse	TGT TCC AG**T GC**T TTG AGC CCG AT	15 s	5 s	30 s	
PPARγC285A	Forward	CAG GGC **GCA** CAG TTT CGC TCC GTG GAG	98 °C	58 °C	72 °C	35
	Reverse	TGT TCC AG**T GC**T TTG AGC CCG AT	15 s	5 s	20 s	

* Mutated nucleotide positions are in bold.

[a] R316A/L326A was generated based on the R316A mutant.

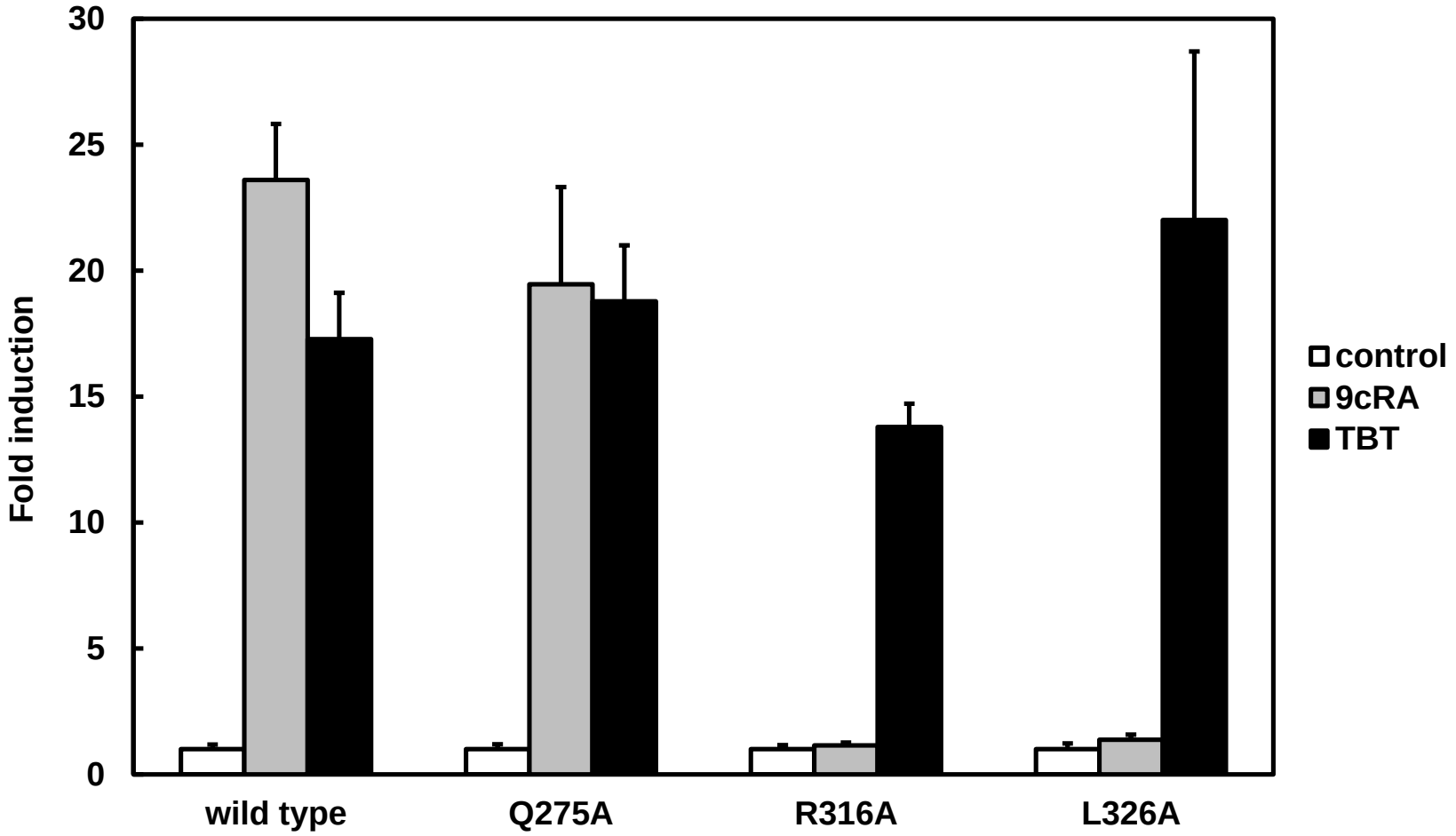

Fig. 1

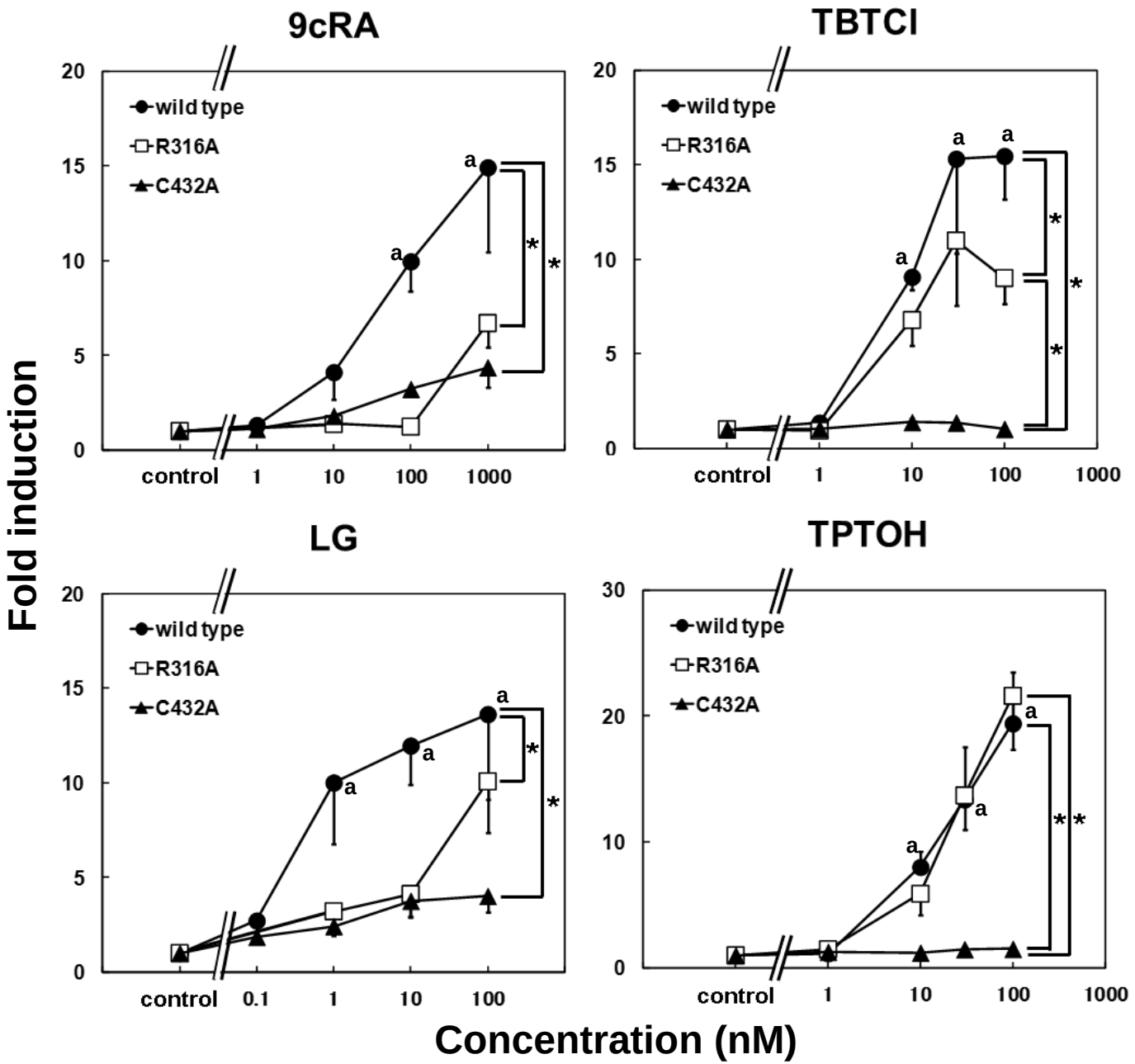

Fig. 2

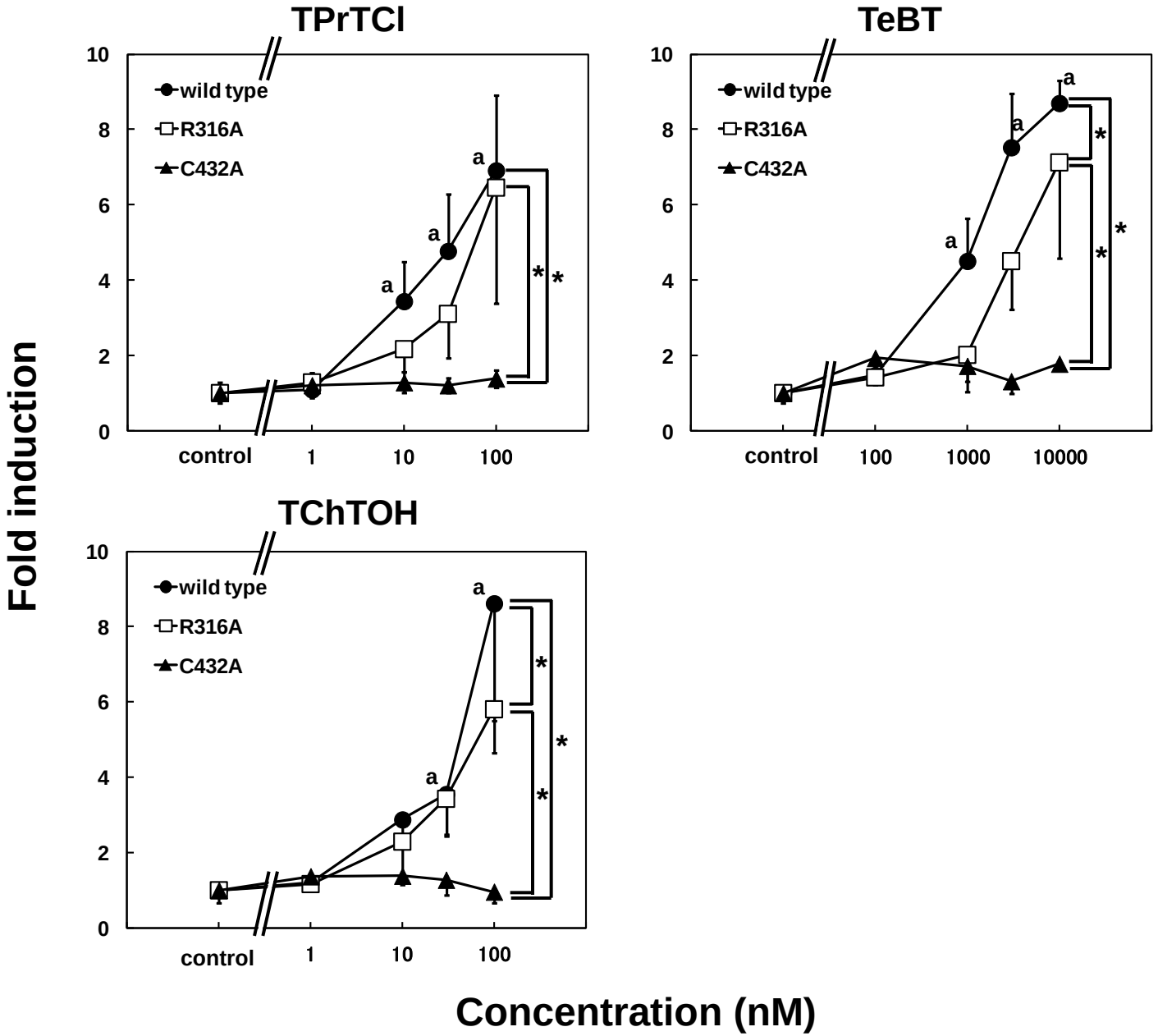

Fig. 3

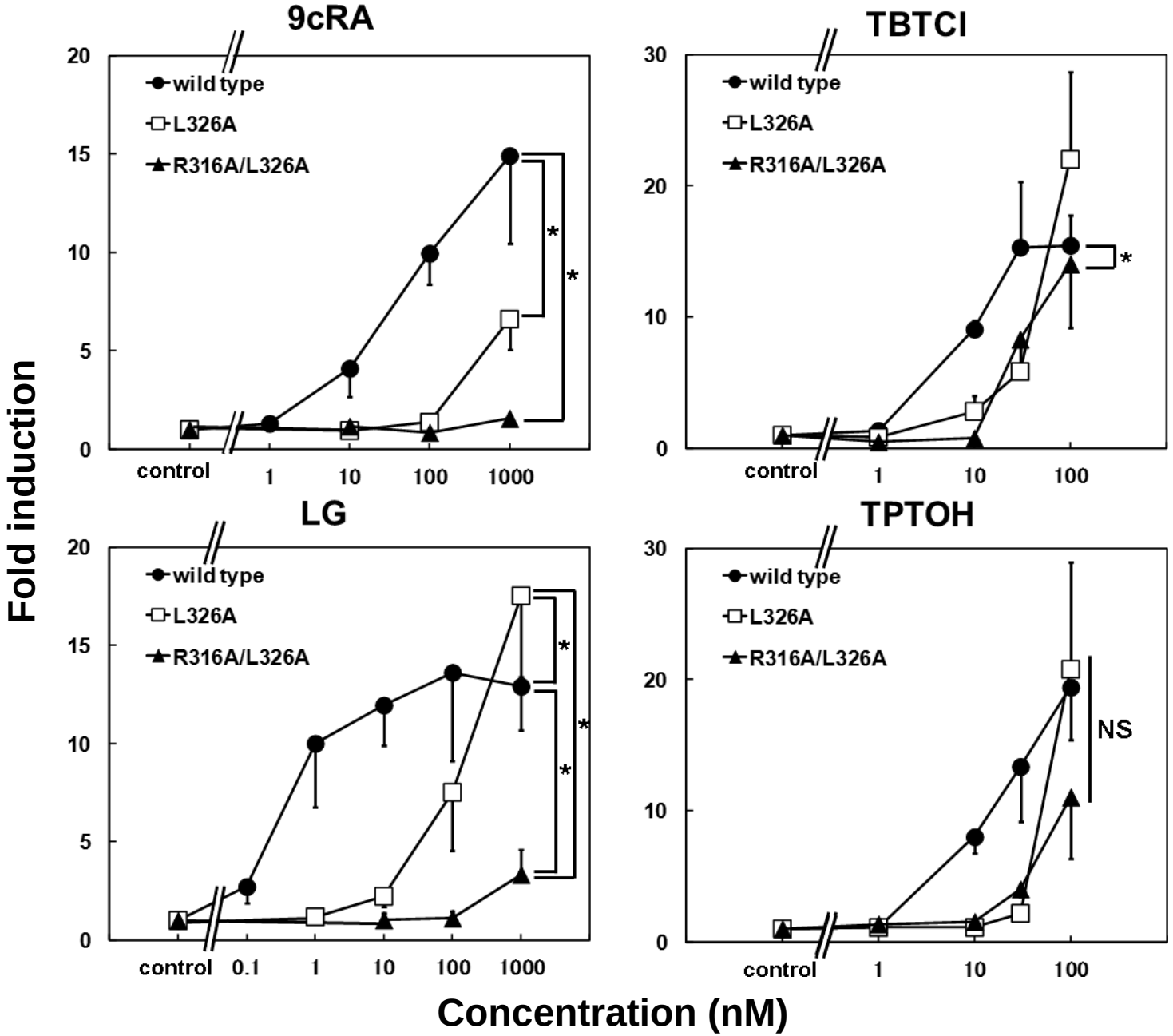

Fig. 4

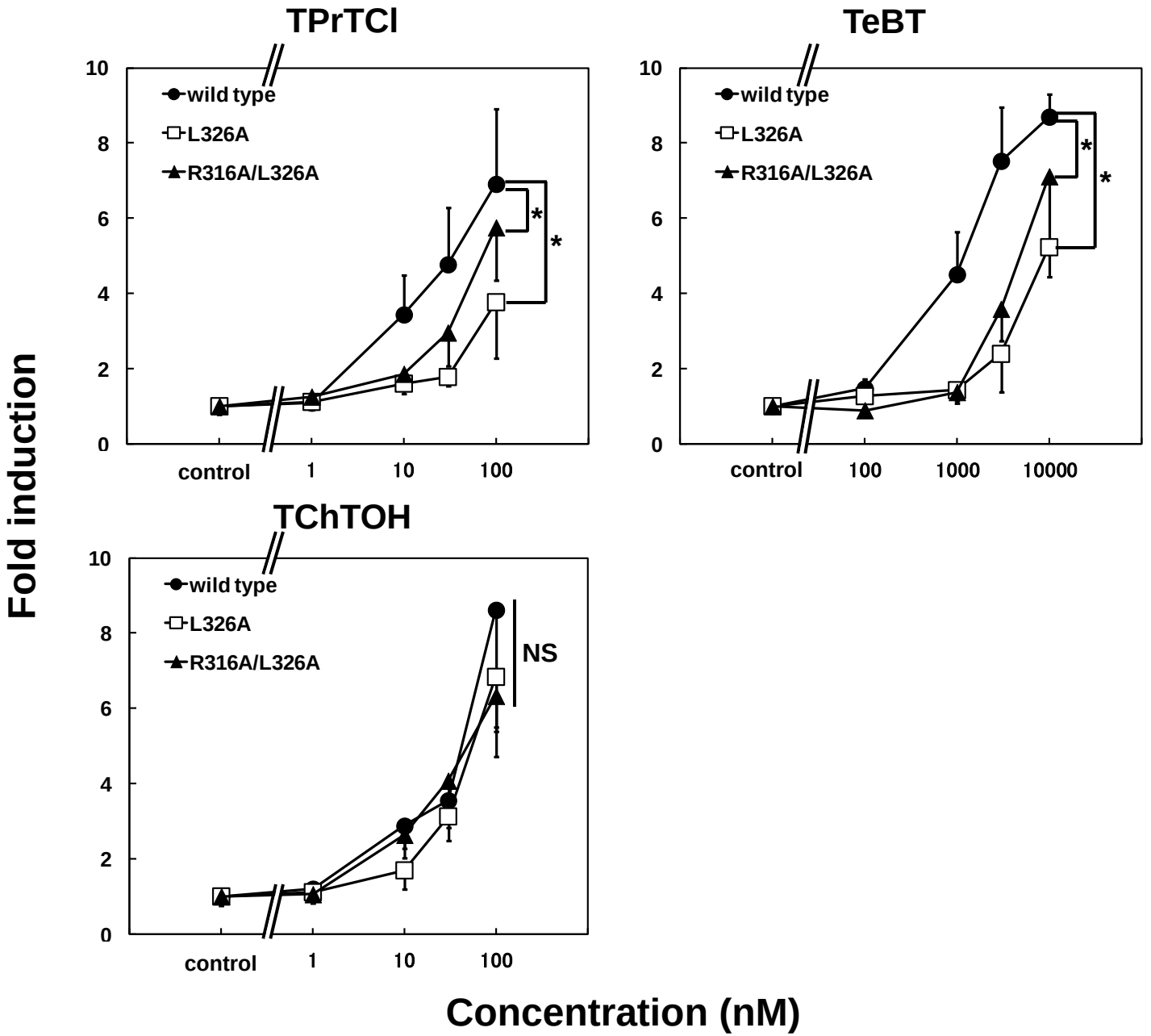

Fig. 5

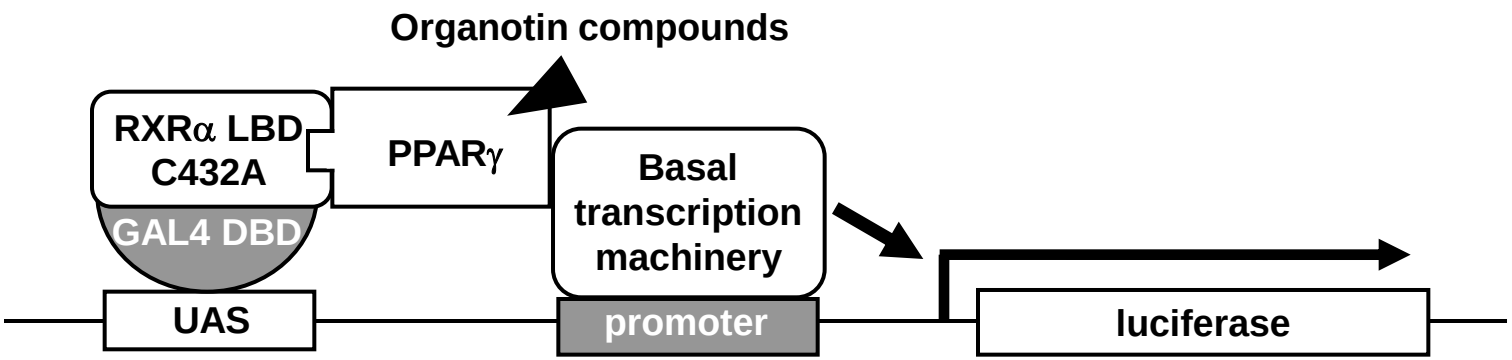

Fig. 6

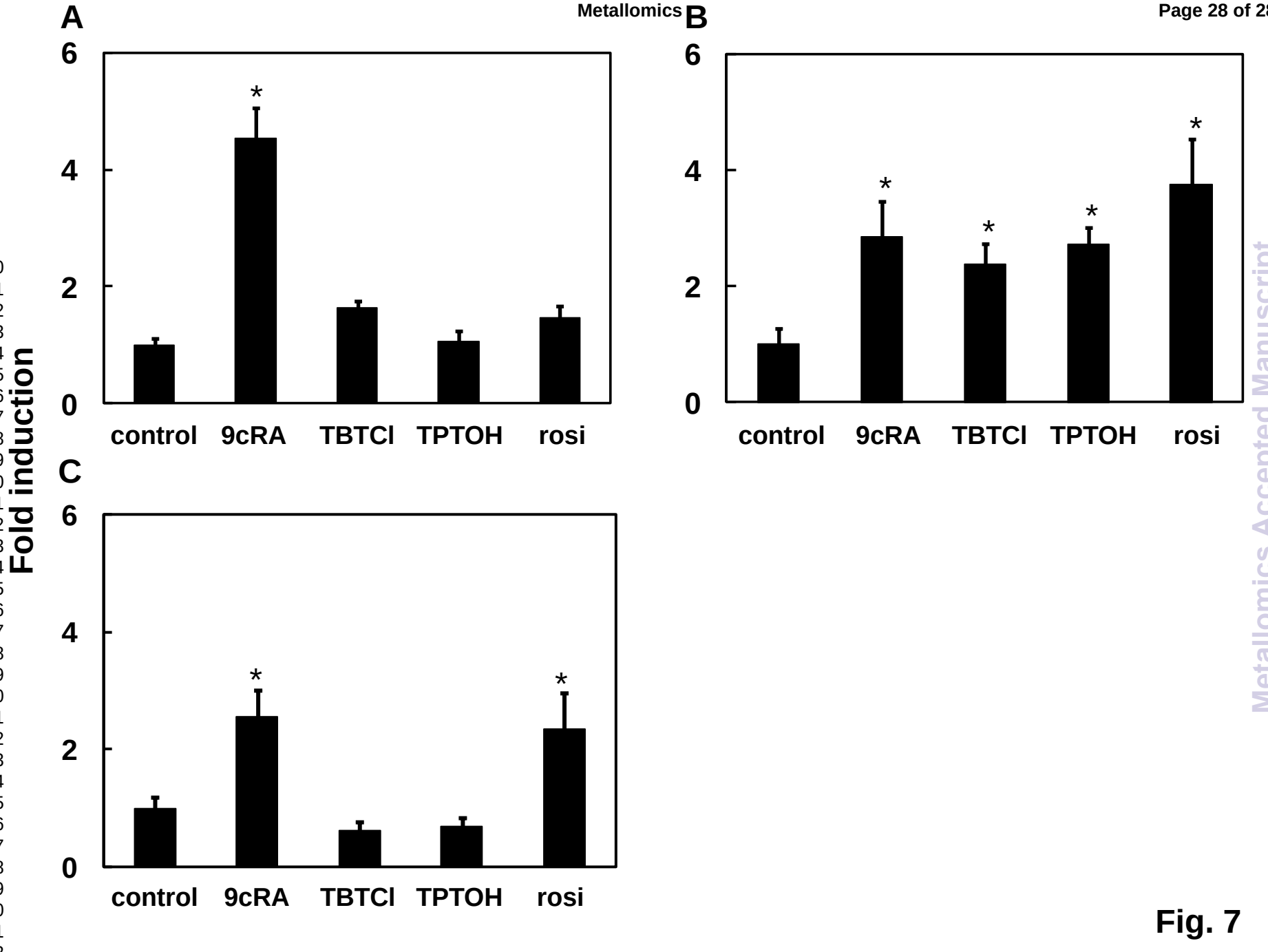

Fig. 7