Niger. J. Physiol. Sci. 26(December 2011) 143 – 149
www.njps.com.ng

Nig. J. Physiol. Sci.

Thyroid hormone receptor-mediated transcription is suppressed by low dose Phthalate

[1,2]Ibhazehiebo K, and [1]Koibuchi N

[1]*Department of Integrative Physiology, Gunma University Graduate School of Medicine, Maebashi, Japan*
[2]*Department of Physiology, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin-City, Nigeria*

Summary: Phthalates are synthetic chemicals used mainly as solvents, additives and plasticizers in polyvinylchloride (PVC) products to increase their flexibility. Phthalate plasticizers are not chemically bound to PVC, so they easily leach into the environment. There is currently heightened concern about potential health risk, especially endocrine disrupting effects associated with the use of these chemicals. We therefore investigated the effects of phthalate on thyroid hormone receptor (TR)-mediated transcription using transient transfection studies and found that low dose phthalate (10^{-7}) M suppressed thyroid hormone (TH)-induced TR-mediated transcription by 30%. We further examined the effect of phthalate on TR-thyroid hormone response element (TRE) binding, and found no dissociation of TR from TRE. Phthalate did not also dissociate coactivator (steroid receptor coactivator-1) from TR neither did it recruit corepressor (nuclear corepressor; NCoR) to TR in the presence of TH. Our results indicate that low phthalate can disrupt TR-mediated gene expression and interfere with TH balance in TH-sensitive organs including the developing brain.

Keywords*:* Phthalate, Thyroid hormone, Thyroid hormone receptor, Transcription.

**Address for correspondence:* dockings22@yahoo.com

Manuscript Accepted: August, 2011

INTRODUCTION

Phthalate are a group of synthetic compounds used in many consumer products, including personal care products, household furnishing, building materials, children's toys, medical devices, dentures, automobiles and cosmetics. They are incorporated into plastics PVC to reduce their rigidity and increase flexibility (Schettler, 2006). More than three million metric tonnes of phthalate are produced annually (Bizzari et al, 2000), and Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate plasticizer in commercial use (Heudorf et al, 2007; Latini G, 2005). Phthalate plasticizers are not chemically bound to PVC, they can leach at a constant rate from plastic products to the environment and consequently, are distributed in the ecosystem (Latini G, 2005), and have been described as the most abundant man-made environmental pollutant (Latini G, 2005).

Humans are exposed to phthalate mainly via ingestion, inhalation and dermal exposure (Adibi et al, 2003, Latini et al, 2003) and the general population exposure to DEHP is estimated to be in the range of 3 - 30ug/kg of body weight/day (excluding occupational exposure, medical exposure and non-dietary ingestions in children) (Shea et al, 2003).

Phthalate are becoming a great environmental concern because of their ubiquitous nature and studies have indicated various reproductive toxicities and carcinogenic potentials. Recent studies suggest that phthalate and their metabolic products could function as antiandrogens especially during the prenatal periods, causing reproductive and developmental abnormalities (Moore et al, 2001). Phthalate have been implicated in reduction in androgen dependant tissues including the seminal vesicles, prostate and epididymis (Mylchreest et al, 2000), reproductive tract malformations (Ema and Miyawaki, 2001), reduced production of testosterone by the fetal testis (Mylchreest et al, 2002).

Specifically, DEHP acting through it metabolite mono-2-ethylhexyl phthalate (MEHP), have been shown to cause decreased testicular weight and tubular atrophy in male rodents (ATSDR, 2002; Gray and Gangolli, 1986), hypoestrogenic anovulatory cycles and polycystic ovaries (Davies et al, 1994), suppression of estradiol production in the ovary in female rodents (Lovekamp-Swan and Davis, 2003).

However, there are currently no studies detailing the disruptive effect of DEHP on TR-mediated gene transactivation at low doses, mechanism of such actions and consequent impact on brain development.

TH is critical for normal brain development and function in animals and humans during the fetal and neonatal periods. They regulate a host of physiological and biochemical processes that converts the fetal brain to that of an adult (Poterfield and Hendrich, 1993) Hypothyroidism especially during the period of brain growth spurt (Dobbing and Sands, 1979) which in humans spans from the third trimester of pregnancy throughout the first two years of life and first three to four weeks in rats and mice after birth, causes abnormal brain development (cretinism) with severe cognitive and / or mental disorders in the offsprings (Koibuchi and Chin, 2000).

TH functions are biologically mediated by TRs which are ligand-dependent transcription factors that are ubiquitously expressed. TR is bound to specific DNA sequence known as TH response element (TRE). When TR binds to a TRE, it interacts with retinoid X receptor (RXR) to form heterodimers, which in turn, binds to a number of coregulators such as corepressors and coactivators in a ligand-dependant manner (Koibuchi, 2008). Disruption of TR-TRE binding or recruitment of corepressors to TR will lead to suppression of TR-mediated transcription and consequent hypothyroid-like conditions which can impair normal brain development.

Our study therefore examines the effect of DEHP on TR-mediated gene expression and possible mechanisms involved.

MATERIALS AND METHODS

Chemicals: Tri-iodothyronine (T3) was purchased from Sigma Chemical Co. (St. Louis USA). DEHP was purchased from AccuStandard Chemicals (New haven, CT, USA) and was >98% pure.

Plasmids: Expression vectors of TRβ1 and glucocorticoid receptor (GR) have been previously described. Briefly, pCMV-GR was generated by inserting GR in to the BamH1/Xho1 sites of pcDNA3 (Invitrogen) (Iwasaki et al, 2001). The luciferase (LUC) reporter constructs, the chick lysozyme (F2)-thymidine kinase (TK)-LUC, are previously described elsewhere (Koibuchi et al, 1999). Briefly, F2 oligonucleotides sequence which contain Hind 111 restriction sites on both ends were cloned into the PT109 vector which contain a viral thymidine kinase promoter coupled to the luciferase coding sequence. 5x upstream activating sequence (UAS)-TK-LUC in the PT109 vector and 2x glucocorticoid response element-LUC reporter in pTAL-LUC were generated by PCR with Nhe1/Xba1 sites of pTAL-Luc (BD Biosciences Clontech, Palo Alto, CA). (Iwasaki et al, 2001). Expression vector of Human SRC-1 were a kind gift from Dr Takeshita (Takeshita et al, 1998). Expression vector of GAL4-DNA-binding domain (DBD)-fused SRC-1-nuclear receptor binding domain (NBD)-1 (aa 595-780) (otherwise described as nuclear receptor-interacting domain) were used (Takeshita et al, 2002). VP16-TRB1-ligand binding domain (LBD) was constructed by inserting PCR-generated fragments inflame downstream of the VP16 activation domain in AASV-VP16. The Gal4-blank, and GAL4-N-CoR (aa 1552-2453) were a kind gift from Dr Takeshita (Takeshita et al, 2002).

Cell culture: Commercially obtained monkey fibroblast-derived CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5 μg/mL penicillin/streptomycin and 10% fetal bovine serum deprived of small lipophilic hormone at 37ºC under a 5% Co2 atmosphere as described previously (Iwasaki et al, 2002).

Transient transfection-based reporter gene assays: Cells were plated in 24-well plates 2 days before transfection by calcium-phosphate precipitation method (Iwasaki et al, 2002). The internal control was Cytomegalovirus-β-galactosidase plasmid. Sixteen to 24 hours after transfection, cells were incubated with fresh medium containing the indicated concentration of ligand and/or DEHP for 24 hours. Cells were then harvested to measure the luciferase activities as described elsewhere (Iwasaki et al, 2002). Total amounts of DNA per well was balanced by adding pcDNA3 plasmids (Invitrogen, San Diego, CA, USA). The LUC activities were normalized to β-galactosidase activity and then calculated as relative LUC activities. All transfection studies were repeated at least twice in triplicate. Data shown represent mean ± S.E.M. of triplicate experiment. The data were analysed using ANOVA.

Trypan blue exclusion: Trypan blue exclusion was previously described (Jiang et al, 2001). Briefly, CV-1 cells were plated in 100-mm diameter plates 2 days before adding DEHP. Each cell was incubated in the presence or absence of 10^{-8} M to 10^{-6} M DEHP. After twenty-four hours, the medium was changed and the cells incubated for 2-5 minutes in a solution of 0.2% trypan blue in phosphate-buffered saline. In each experiment, non-viable cells (colorless) and viable cells (stained blue) were counted. The data were analyzed by ANOVA.

Liquid Chemiluminescent DNA pull down Assay (LCDPA): Liquid chemiluminescent DNA pull down assay to examine nuclear receptor-DNA binding in solution was previously described (Iwasaki et al, 2008). Briefly, a glutathione S-transferase (GST)-fused TH receptor (GST-TR) bound to glutathione-

Sepharose beads was incubated with a digoxigenin (DIG)-labeled double–stranded DNA fragment containing a TH response element (TRE) in protein-DNA binding buffer. After extensive washing, protein-DNA binding on beads is detected using anti-DIG antibody conjugated to alkaline phosphatase, which is then measured by a chemiluminescent reaction using a luminometer. We perform LCDPA at least three times and data shown represent mean ± S.E.M. The data were analyzed by ANOVA.

RESULTS

Low Dose Phthalate suppresses TR-mediated gene expression.

We examined the effect of DEHP on TR-mediated gene transcription using the transient transfection-based reporter gene assay in CV-1 cell (Figure 2). Suppression of TR-mediated transcription on the F2-TRE-LUC was observed at doses ranging from 10^{-6} M to 10^{-7} M. 30% suppression was observed at 10^{-7} M (Figure 2). The effects of DEHP suppression was not dose dependent suggesting that DEHP did not competitively inhibit T3 action. Also, DEHP effect was not as a result of cell death as confirmed by Trypan blue exclusion (data not shown). Also, DEHP did not affect GR-mediated transcription (Figure 3) indicating that the suppression was TR-specific.

DEHP did not inhibit SRC-1 binding to TR.

We examined the effect of DEHP on binding between TR and SRC-1 in CV-1 cells using mammalian two-hybrid assay. In this assay, the interaction between SRC-1-NBD-1 and TRβ1-LBD with or without T3 and DEHP was investigated. The NBD-1 of SRC-1 was fused to the Gal-4-DNA binding domain, and the LBD of TRβ1 was fused to VP16 transactivation domain. VP16 alone had no significant increase on transcription (Figure 4; columns 3), and transactivation mediated by Gal4-SRC-1-NBD-1 and VP16-TRβ1-LBD proceeded in the presence of T3 (Figure 4; column 4).

Transcriptional activation caused by SRC-1-NBD-1 and VP16-TRβ1-LBD interaction with T3 was not significantly affected by DEHP at concentrations of 10^{-11} M and 10^{-6} M (Figure 4; columns 5and 6). These results indicate that DEHP did not inhibit the interaction between SRC-1 and TRβ1-LBD.

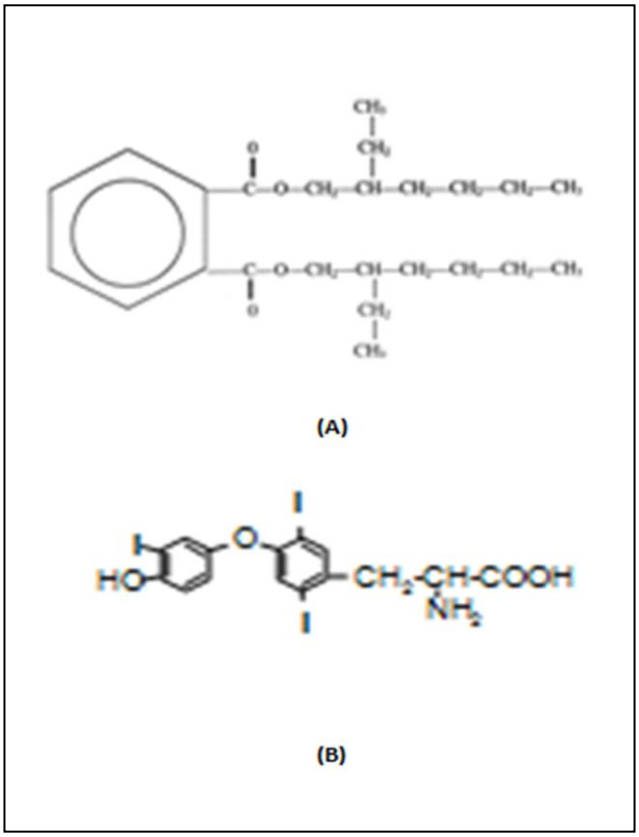

Fig 1:
Structure of (A) DEHP and (B) T_3

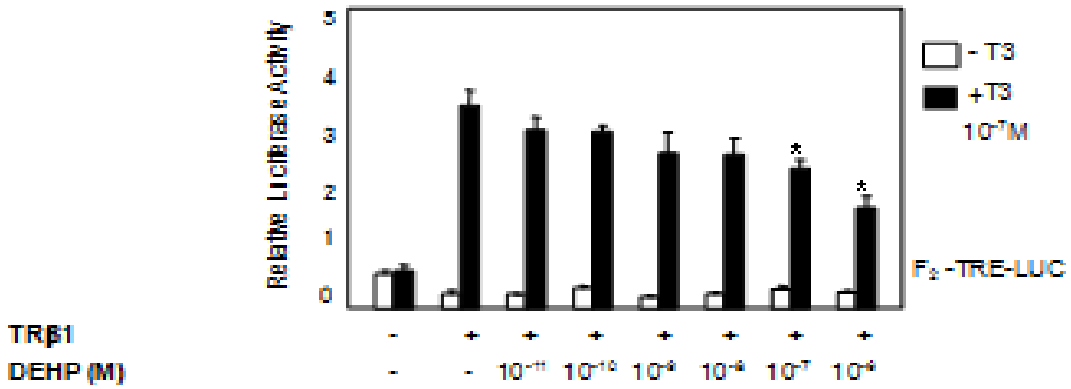

Figure 2:
Low Dose Phthalate suppresses TR-mediated transactivation.
Expression plasmids encoding TRβ1 (10 ng) were cotransfected with F2-TK-LUC (100 ng) into CV-1 cells. Cells were incubated with or without 10^{-7} M T3 and indicated amount of DEHP. Total amounts of DNA for each well were balanced by adding vector pcDNA3. Data represent mean ± S.E.M. of experiments performed in triplicate. *, statistically significant $p<0.05$ by ANOVA versus TRβ1 (+), T3 (+), and DEHP (-).

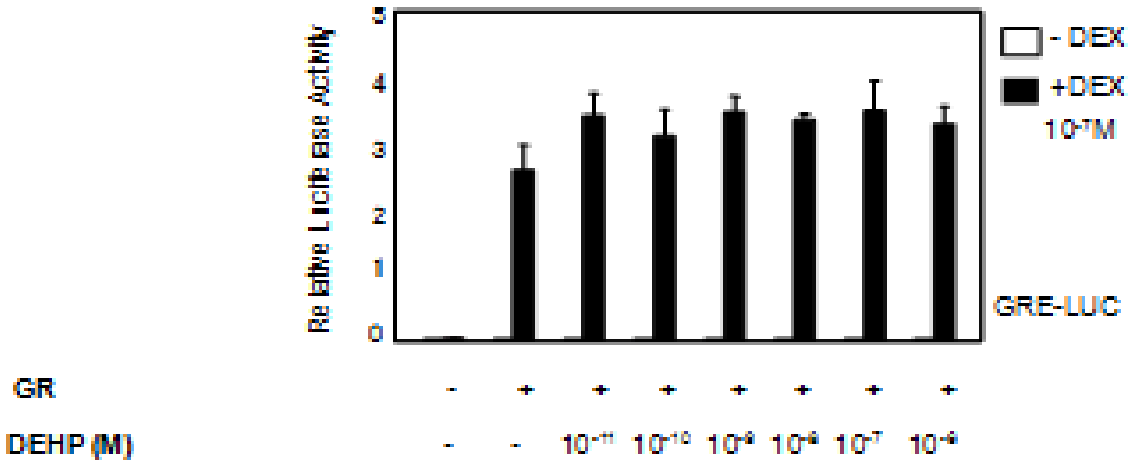

Figure 3:
Phthalate did not suppress GR-mediated transcription
Expression plasmids encoding GR (10 ng) were cotransfected with glucocorticoid response element (GRE)-LUC reporter plasmids (100 ng) into CV-1 cells. Cells were cultured in the absence or presence of dexamethasone (Dex) (10^{-7} M) and indicated concentrations of DEHP. Total amounts of DNA for each well were balanced by adding vector pcDNA3. Data represent mean ± S.E.M. of experiments performed in triplicate. No statistical significance was uncovered by ANOVA.

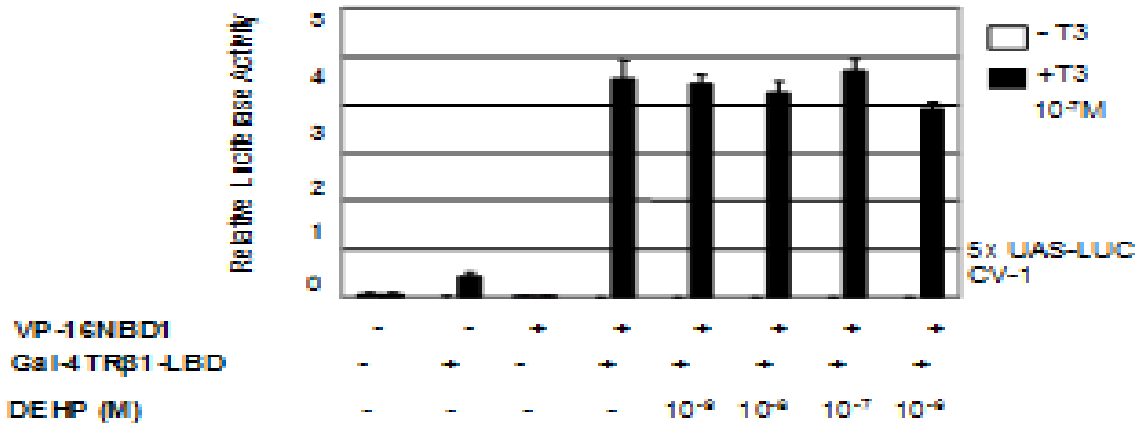

Figure 4.
DEHP did not inhibit SRC-1 binding to TR.
Expression plasmids encoding Gal4-DBD-fused SRC-1-NBD-1 (10 ng) were cotransfected with VP16-constructs (50 ng) and 5xUAS-TK-LUC-reporter plasmids (170 ng) into CV-1 cells. Cells were incubated with or without T3 (10^{-7} M) and indicated concentrations of DEHP. Total amounts of DNA for each well were balanced by adding vector pcDNA3. Data represent mean ± S.E.M. of experiments performed in triplicate. No statistical significance was uncovered by ANOVA.

DEHP did not recruit N-CoR to TR.
We examined the effect of HBCD on binding between N-CoR and TR in CV-1 cells using mammalian two hybrid assay. Gal4-N-CoR or VP16-TRβ1 and 5x UAS-TK-LUC were cotransfected into CV-1 cells. Gal4-N-CoR and VP16-TRβ1 precedes the transcriptional activity (Figure 5, column 4) in the absence of T3, while no activation was observed with T3. Transcriptional activities were not markedly altered in the presence of 10^{-11} M and 10^{-6} M DEHP regardless of T3 10^{-7} M (Figure 5, column 5-6), neither was there any significant dissociation of N-CoR from TR in the absence of T3 (Figure 5) indicating that PBDE may not recruit N-CoR to TR in the presence of T3.

DEHP did not dissociate TR from TRE.

We performed liquid chemiluminescent DNA pull down assay to examine the effect of DEHP on TR binding to TRE. We have confirmed previously that the results of this assay were compatible to those of

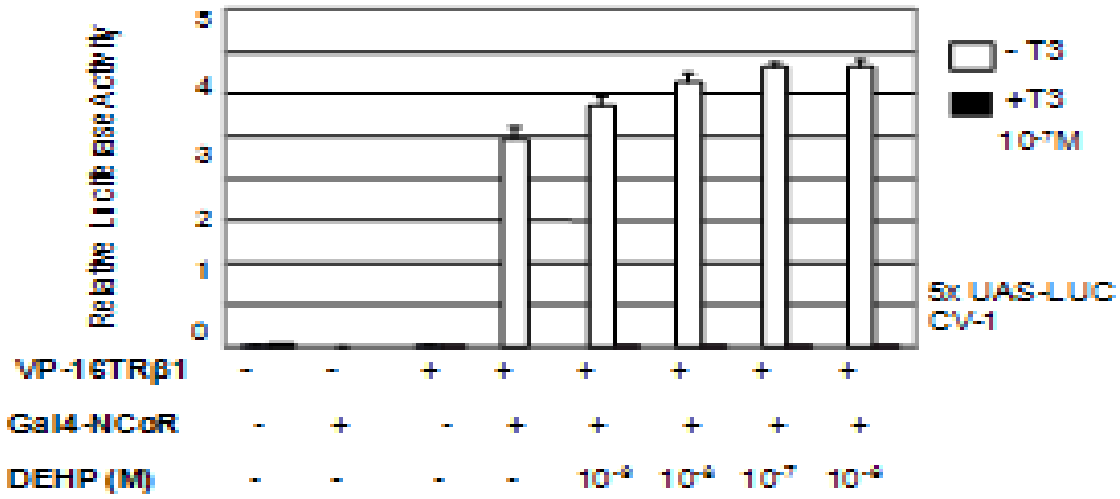

Figure 5.

DEHP did not recruit N-CoR to TR in the absence of T3 in CV-1 cells. Expression plasmids harboring Gal4-DBD-fused N-CoR (100 ng) were cotransfected with VP16- TRβ1-LBD (50 ng) and 5x UAS-TK-LUC (100 ng) into CV-1 cells with or without T3 (10^{-7} M) and/or indicated amount of DEHP. Total amounts of DNA for each well were balanced by adding vector pcDNA3. No statistical significance was determined by ANOVA.

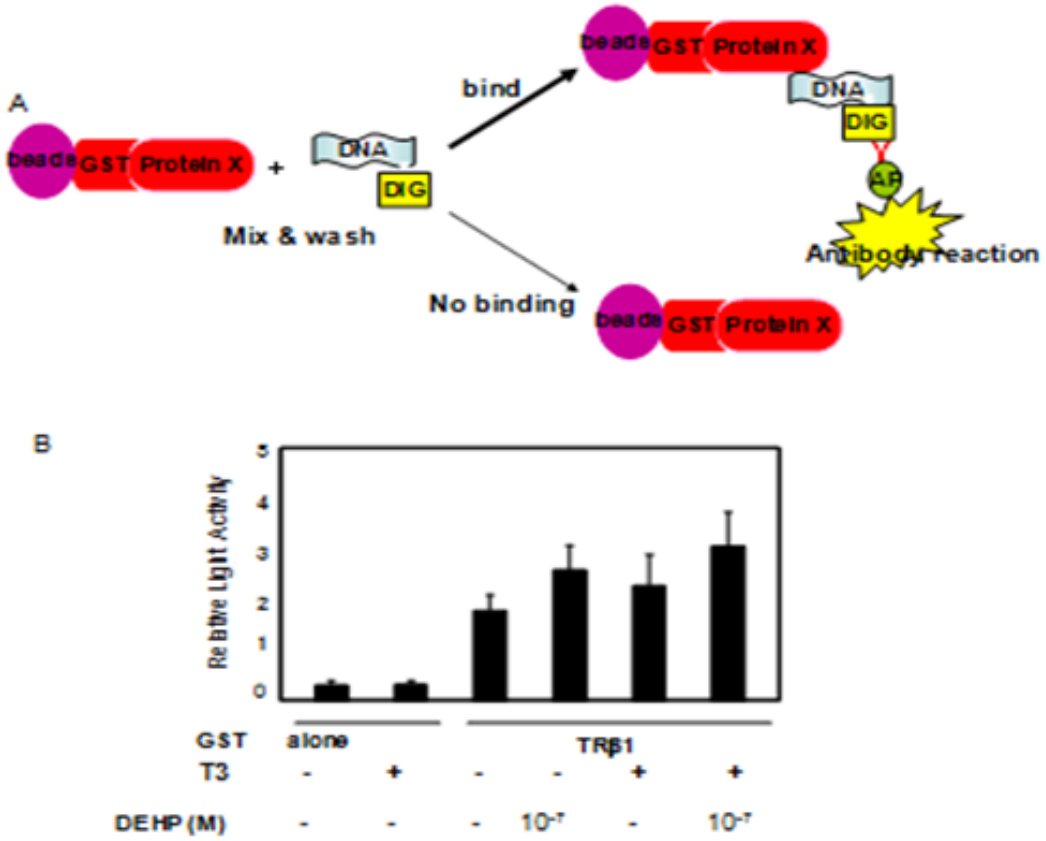

Figure 6.

DEHP did not dissociate TR from TRE. GST-TRβ1 bound to sepharose bead is incubated with DIG-F2 containing TRE in protein-DNA binding buffer with or without T3 (10^{-6} M) and 10^{-7} M DEHP. Data represent mean ± S.E.M. of experiments performed in triplicate. No statistical significance was uncovered by ANOVA.

Electrophoretic Mobility Shift Assay (Iwasaki et al, 2008). DEHP 10^{-6} M did not dissociate TR from TRE in the presence of T3 10^{-6}M (Figure 6B, column 6), though there was a tendency to enhanced TR-TRE binding. This indicates that the suppression of TR-mediated transcription by DEHP was not due to dissociation of TR complex from the TRE.

DISCUSSION

Our study clearly shows that low dose DEHP induces suppression of TR-mediated transcription in the presence of T3 and may consequently interfere with normal brain development and function especially during the critical period of brain development.

Suppression of TR-mediated transcription by DEHP was not dose dependent (Figure 2) suggesting that such effect is not as a result of increased billiary excretion of T3 due to competitive inhibition by DEHP binding to TR. These findings are consistent with previous studies that Polychlorinated biphenylether (PCB), another endocrine disruptor can interfere with TH homeostasis (Miyazaki et al. 2008). DEHP suppressive actions was not as a result of cell death because trypan blue exclusion showed that DEHP did not affect cell viability under our culture conditions (data not shown).The suppressive effect shown by DEHP was TR-specific because no suppression was seen in the presence of GR and DEHP (Figure 3).

Our initial hypothesis was that DEHP effect could be by dissociation of the coactivator complex from TR or through the recruitment of a corepressor complex to TR since TR function was regulated in a ligand-dependent manner involving nuclear cofactors (coactivators or corepressors). However, DEHP did not dissociate SRC-1 from TR, neither did it recruit complexes containing N-CoR to TR, indicating mechanisms other than cofactors interaction could be involved. Also, another endocrine disruptor Bisphenol A have been shown to recruit corepressor complexes containing N-CoR to TR thereby suppressing its action (Moriyama et al.2002), however, in our study DEHP did not recruit N-CoR to TR in the absence of T3 , thus the mechanism by which DEHP affect TR action may be different from that of Bisphenol A.

Liquid chemiluminescent DNA pull down assay also did not show any dissociation between TR and TRE in the presence of DEHP and T3, although a tendency towards enhanced TR-TRE binding was observed indicating that DNA-protein interaction involving response elements may not be involved in DEHP action and that DEHP may act indirectly on TR to suppress gene expression.

TH is essential for normal brain growth and function and conditions of hypothyroidism especially during the perinatal period have been known to induce cretinism with severe cognitive and/or mental disorders in the offsprings (Koibuchi et al. 2000). Since TH also tightly regulate fundamental gene expression both directly and indirectly in vast brain region (Koibuchi et al, 1999, 2000), The inhibitory effects of DEHP may disrupt normal brain development via TH-dependent gene regulations. More studies are however required to further elucidate the mechanism by which DEHP suppresses TR-mediated gene expression, and a plausible mechanistic path to further explore could be the effect of DEHP on chromatin modifications.

In conclusion, our study shows that DEHP suppresses TR-mediated transcription thereby disrupting TH homeostasis and may consequently interfere with normal brain development and function. We hope that this work will further underscore the need for regulation of DEHP in consumer products.

REFERENCES

Adibi, J.J., Perera, F.P., Jedrychowski, W., et al. (2003). Prenatal exposures to phthalates among women in New York city and Krakow, Poland. Environ. Health Perspect. 111:1719-1722.

ATSDR, (2002). Toxicological profile for Di(2-ethylhexyl)phthalate. Atlanta, GA; Agency for toxic substances and disease registry.

Bizzari, S., Oppenberg, B., Iskikawa,Y. (2000). Platicizers. Chemical Economics Handbook. SRI international, Palo Alto, CA, USA.

Davis, B.J, Maronpot, R.R, Heindel, J.J. (1994). Di-(2-ethylhexyl)phthalate suppresses estradiol and ovulation in cycling rats. Toxicol.Appl.Pharmacol. 128:216-223.

Dobbing, J., and Sands, J. (1979). Comparative aspects of the brain growth spurt. Early Hum Dev 3:79-83.

Ema, M., Miyawaki, E. (2001). Adverse effects on development of the reproductive system in male offspring of rats given monobutyl phthalate, a metabolite of dibutyl phthalate, during late pregnancy. Reprod. Toxicol. 15:189-194.

Gray, T.J.B., Gangolli, S.D. (1986). Aspects of the testicular toxicity of phthalate-esters. Environ. Health Perspect. 65:229-235.

Haddow, J.E., Palomoki, G.E., Allan, W.C., et al (1999). Maternal thyroid deficiency during pregnancy and subsequent development of the child. New Engl. J. Med. 341:549-555.

Heurdorf, U., Mersch-Sundermann, V., Angerer, J. (2007). Phthalate: Toxicology and exposure. Int. J. Hyg. Environ. Health. 210:623-634.

Iwasaki, T., Chin, W.W., Ko., L. (2001). Identification and characterization of RRM-containing coactivator activator (CoAA) as TRBP-interacting protein and its splice variant as a coactivator modulator (CoAM). J. Biol. Chem. 276:33375-33383.

Iwasaki, T., Miyazaki, W. Takeshita, A. Kuroda Y., et al. (2002). Polychlorinated biphenyls suppress thyroid hormone –induced transactivation. Biochem. Biophys. Res. Commun. 298:384-388.

Iwasaki, T., Miyazaki, W., Rokutanda, N., et al. (2008). Liquid chemiluminescent DNA pull-down assay to measure nuclear receptor-DNA binding in solution. Biotechniques 45:445-448.

Jiang, D., Sullivan, P.G.., Sensi, S.L., et al. (2001). Zn (2+) induces permeability transition pore opening and release of pro-apoptotic peptodes from neuronal membrane. J .Biol. Chem. 276:47524-47529.

Koibuchi, N., Liu, Y., Fukuda, H. et al., (1999). ROR augments thyroid hormone receptor-mediated transcriptional activation. Endocrinology 140: 1356-1364.

Koibuchi, N., Chin, W.W. (2000). Thyroid hormone action and brain development. Trends. Endocrinol. Metab. 11:123-128.

Koibuchi, N. (2008). The role of thyroid hormone on Cerebellar development. Cerebellum 7:530-533.

Latini, G., De Felice, C., Presta, G. et al., (2003). Exposure to Di-(2-ethylhexyl)-phthalate in humans during pregnancy: a preliminary report. Biol Neonate. 83:22-24.

Latini, G. (2005). Monitoring phthlalte exposure in humans. Clinica Chimica Acta. 361:20-29.

Lovekamp-Swan, T., Davis, B.J. (2003). Mechanisms of phthalate ester toxicity in the female reproductive biosystem. Environ. Health Perspect. 111:139-145.

Miyazaki, W., Iwasaki, T., Takeshita, A., et al., (2008). Identification of the functional domain of thyroid hormone receptor responsible for polychlorinated Biphenyl-mediated suppression of its action in vitro. Environ. Health Perspect. 116:1231-1236.

Moore, R.W., Rudy, T.A., Lin, T.M., et al. (2001). Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer di(2-ethylhexyl) phthalate. Environ. Health Perspect. 109:229-237.

Moriyama, K., Tagami, T., Akamizu, T., et al (2002). Thyroid hormone action is disrupted by bisphenol A as an antagonist. J Clin Endocrinol Metab 87: 5158-5190.

Mylchreest, E., Wallace, D.G., Cattley, R.C., et al (2000). Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. Toxicol Sci 55:143-151.

Mylchreest, E., Sar, M., Wallace, D.G.., et al. (2002). Fetal testosterone insufficiency and abnormal proliferation of leydig cells and gonocytes in rats exposed to di(n-butyl) phthalate. Reprod Toxicol 16:19-28.

Potterfield, S.P., Hendrich, C.E., (1993). The role of thyroid hormones in prenatal and neonatal neurologic development: current perspectives. Endocr Rev 14:94-106.

Schettler, T. (2006). Human exposure to phthalate via consumer products. Int J Androl 29:134-139.

Shea, K.M., and American Academy of Pediatrics Committee on Environmental health. (2003). Pediatric exposure and potential toxicity of phthalate plasticizers. Peadiatrics 111:1467-1474.

Takeshita, A., Yen, P.M., Ikeda, M, et al. (1998). Thyroid hormone response elements differentially modulate the interactions of thyroid hormone receptor with two receptor binding domain in the steroid receptor Coactivator-1. J Biol Chem 273:21554-21562.

Takeshita, A., Taguchi, M., Koibuchi, N., et al. (2002). Putative role of the orphan nuclear receptor SXR (Steroid and xenobiotic receptor) in the mechanism of CYP3A4 inhibition by xenobiotics. J Biol Chem 277:32453-32458.