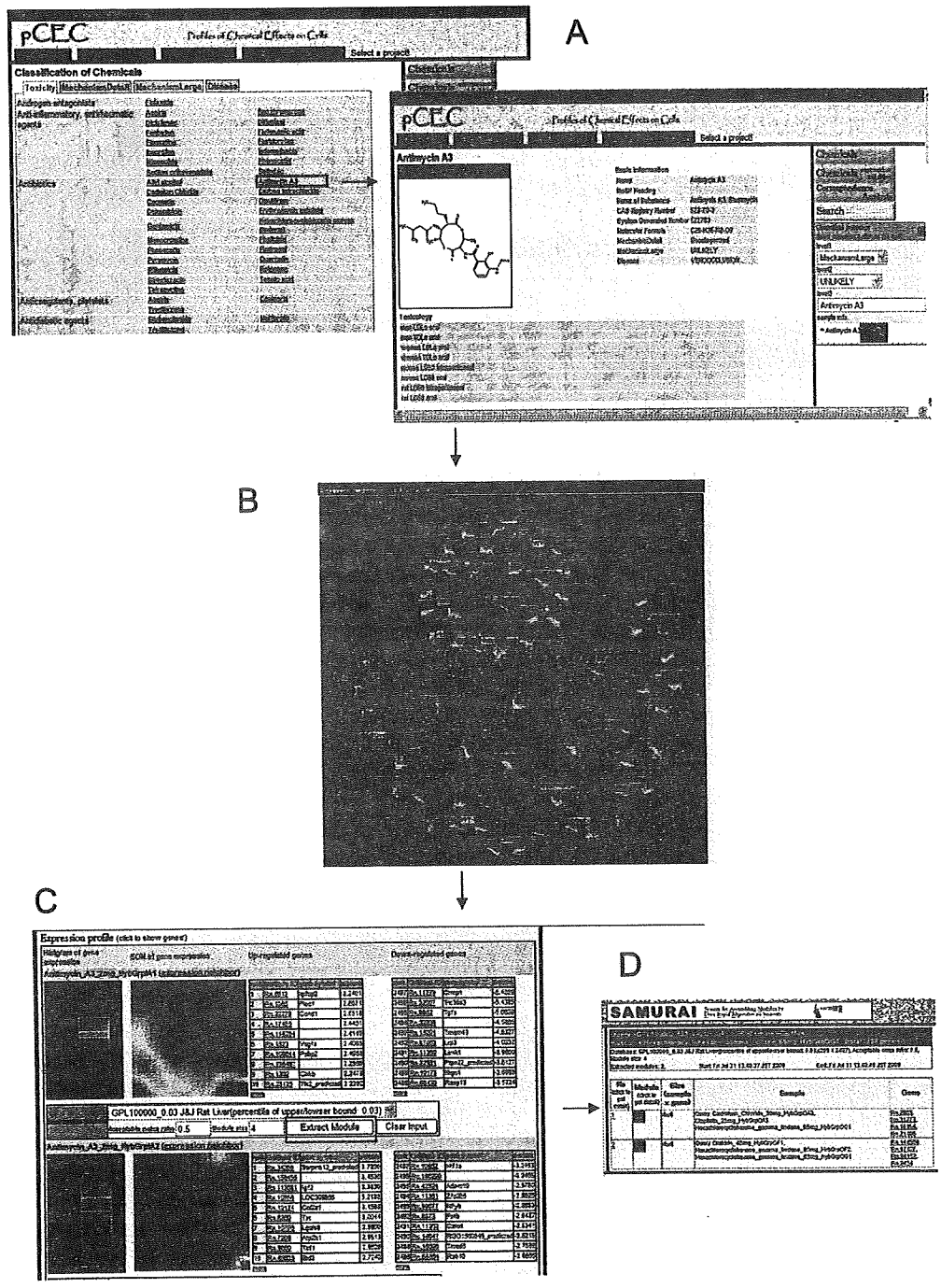

Fig. 5. Chemical selector. This query option is categorized by the chemical at three levels. The user chooses options in Levels 1 and 2 and chooses the desired chemical in Level 3.
Level 1 indicates the categories of the different types of chemical effects, which are parts of the process during apparent toxic effects and disease outcomes from the first target on the cellular event as shown in Fig. 1. Level 2 indicates more detailed categories of the mechanism of action and toxicity pathways than those of level 1. Level 3 indicates chemicals that are related to the structure of the mother chemical.

ACKNOWLEDGMENTS

We thank Prof. Richard E. Peterson (the University of Wisconsin-Madison) for his comments on this work. This work was supported by an Environmental Risk Program of the National Institute for Environmental Studies.

REFERENCES

Aardema, M.J. and MacGregor, J.T. (2002): Toxicology and genetic toxicology in the new era of "toxicogenomics": impact of "-omics" technologies. Mutat. Res., **499**, 13-25.

Ahlers, J., Stock, F. and Werschkun, B. (2008): Integrated testing and intelligent assessment-new challenges under REACH. Environ. Sci. Pollut. Res. Int., **15**, 565-572.

Andersen, M.E. and Krewski, D. (2009): Toxicity testing in the 21st century: bringing the vision to life. Toxicol. Sci., **107**, 324-330.

Barrett, T., Suzek, T.O., Troup, D.B., Wilhite, S.E., Ngau, W.C., Ledoux, P., Rudnev, D., Lash, A.E., Fujibuchi, W. and Edgar, R. (2005): NCBI GEO: mining millions of expression profiles--database and tools. Nucleic. Acids. Res., **33**, D562-566.

Barrett, T., Troup, D.B., Wilhite, S.E., Ledoux, P., Rudnev, D., Evangelista, C., Kim, I.F., Soboleva, A., Tomashevsky, M. and Edgar, R. (2007): NCBI GEO: mining tens of millions of expression profiles--database and tools update. Nucleic. Acids. Res., **35**, D760-765.

Benigni, R., Netzeva, T.I., Benfenati, E., Bossa, C., Franke, R., Helma, C., Hulzebos, E., Marchant, C., Richard, A., Woo, Y.T. and Yang, C. (2007): The expanding role of predictive toxicology: an update on the (Q)SAR models for mutagens and carcinogens. J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev., **25**, 53-97.

Bushnell, P.J., Boyes, W.K., Shafer, T.J., Bale, A.S. and Benignus, V.A. (2007): Approaches to extrapolating animal toxicity data on organic solvents to public health. Neurotoxicology, **28**, 221-226.

Fentem, J., Chamberlain, M. and Sangster, B. (2004): The feasibility of replacing animal testing for assessing consumer safety: a suggested future direction. Altern. Lab. Anim., **32**, 617-623.

Fostel, J.M. (2008): Towards standards for data exchange and integration and their impact on a public database such as CEBS (Chemical Effects in Biological Systems). Toxicol. Appl. Pharmacol., **233**, 54-62.

Guyton, K.Z., Kyle, A.D., Aubrecht, J., Cogliano, V.J., Eastmond, D.A., Jackson, M., Keshava, N., Sandy, M.S., Sonawane, B., Zhang, L., Waters, M.D. and Smith, M.T. (2009): Improving prediction of chemical carcinogenicity by considering multiple mechanisms and applying toxicogenomic approaches. Mutat. Res., **681**, 230-240.

Judson, R., Richard, A., Dix, D., Houck, K., Elloumi, F., Martin, M., Cathey, T., Transue, T.R., Spencer, R. and Wolf, M. (2008): ACToR--Aggregated Computational Toxicology Resource. Toxicol. Appl. Pharmacol., **233**, 7-13.

Judson, R., Richard, A., Dix, D.J., Houck, K., Martin, M., Kavlock, R., Dellarco, V., Henry, T., Holderman, T., Sayre, P., Tan, S., Carpenter, T. and Smith, E. (2009): The toxicity data landscape for environmental chemicals. Environ. Health Perspect., **117**, 685-695.

Kavlock, R.J., Ankley, G., Blancato, J., Breen, M., Conolly, R., Dix, D., Houck, K., Hubal, E., Judson, R., Rabinowitz, J., Richard, A., Setzer, R.W., Shah, I., Villeneuve, D. and Weber, E. (2008): Computational toxicology--a state of the science mini review. Toxicol. Sci., **103**, 14-27.

Krewski, D., Andersen, M.E., Mantus, E. and Zeise, L. (2009): Toxicity testing in the 21st century: implications for human health risk assessment. Risk Anal., **29**, 474-479.

Lambert, C.B., Spire, C., Renaud, M.P., Claude, N. and Guillouzo, A. (2009): Reproducible chemical-induced changes in gene expression profiles in human hepatoma HepaRG cells under various experimental conditions. Toxicol. In Vitro, **23**, 466-475.

Luo, F., Khan, L., Bastani, F., Yen, I.L. and Zhou, J. (2004): A dynamically growing self-organizing tree (DGSOT) for hierarchical clustering gene expression profiles. Bioinformatics, **20**, 2605-2617.

Masciocchi, J., Frau, G., Fanton, M., Sturlese, M., Floris, M., Pireddu, L., Palla, P., Cedrati, F., Rodriguez-Tomé, P. and Moro, S. (2009): MMsINC: a large-scale chemoinformatics database. Nucleic. Acids. Res., **37**, D284-290.

Mattingly, C.J. (2009): Chemical databases for environmental health and clinical research. Toxicol. Lett., **186**, 62-65.

Maxwell, G., Aleksic, M., Aptula, A., Carmichael, P., Fentem, J., Gilmour, N., Mackay, C., Pease, C., Pendlington, R., Reynolds, F., Scott, D., Warner, G. and Westmoreland, C. (2008): Assuring consumer safety without animal testing: a feasibility case study for skin sensitisation. Altern. Lab. Anim., **36**, 557-568.

National Research Council (2007): Toxicity Testing in the 21st Century: A Vision and a Strategy. National Academies Press, Washington, DC.

Okada, Y., Okubo, K., Fujibuchi, W. and Horton, P. (2007): Exhaustive Search Method of Gene Expression Modules and Its Application to Human Tissue Data. IAENG International Journal of Computer Science, **34**, 119-126.

Schmidt, U., Struck, S., Gruening, B., Hossbach, J., Jaeger, I.S., Parol, R., Lindequist, U., Teuscher, E. and Preissner, R. (2009): SuperToxic: a comprehensive database of toxic compounds. Nucleic. Acids. Res., **37**, D295-299.

Schneider, K., Schwarz, M., Burkholder, I., Kopp-Schneider, A., Edler, L., Kinsner-Ovaskainen, A., Hartung, T. and Hoffmann, S. (2009): "ToxRTool", a new tool to assess the reliability of toxicological data. Toxicol. Lett., **189**, 138-144.

Shoemaker, R.H. (2006): The NCI60 human tumour cell line anticancer drug screen. Nat. Rev. Cancer, **6**, 813-823.

Tomasulo, P. (2005): ITER- International Toxicity Estimates for Risk, new TOXNET database. Med. Ref. Serv. Q, **24**, 55-66.

Törönen, P., Kolehmainen, M., Wong, G. and Castrén, E. (1999): Analysis of gene expression data using self-organizing maps. FEBS Lett., **451**, 142-146.

Wang, R.Y. (2008): Medical toxicology and public health-update on research and activities at the Centers for Disease Control and Prevention and the Agency for Toxic Substances and Disease Registry. J. Med. Toxicol., **4**, 143-144.

Waters, M. and Jackson, M. (2008): Databases applicable to quantitative hazard/risk assessment--towards a predictive systems toxicology. Toxicol. Appl. Pharmacol., **233**, 34-44.

Waters, M., Stasiewicz, S., Merrick, B.A., Tomer, K., Bushel, P., Paules, R., Stegman, N., Nehls, G., Yost, K.J., Johnson, C.H., Gustafson, S.F., Xirasagar, S., Xiao, N., Huang, C.C., Boyer, P., Chan, D.D., Pan, Q., Gong, H., Taylor, J., Choi, D., Rashid, A., Ahmed, A., Howle, R., Selkirk, J., Tennant, R. and Fostel, J. (2008): CEBS--Chemical Effects in Biological Systems: a public data repository integrating study design and toxicity data with microarray and proteomics data. Nucleic. Acids. Res., **36**, D892-900.

Waters, M.D. and Fostel, J.M. (2004): Toxicogenomics and systems toxicology: aims and prospects. Nat. Rev. Genet., **5**, 936-948.

Waters, M.D., Olden, K. and Tennant, R.W. (2003): Toxicogenomic approach for assessing toxicant-related disease. Mutat. Res., **544**, 415-424.

Williams-DeVane, C.R., Wolf, M.A. and Richard, A.M. (2009a): DSSTox chemical-index files for exposure-related experiments in ArrayExpress and Gene Expression Omnibus: enabling toxico-chemogenomics data linkages. Bioinformatics, **25**, 692-694.

Williams-Devane, C.R., Wolf, M.A. and Richard, A.M. (2009b): Toward a public toxicogenomics capability for supporting predictive toxicology: survey of current resources and chemical indexing of experiments in GEO and ArrayExpress. Toxicol. Sci., **109**, 358-371.

Woodruff, T.J., Zeise, L., Axelrad, D.A., Guyton, K.Z., Janssen, S., Miller, M., Miller, G.G., Schwartz, J.M., Alexeeff, G., Anderson, H., Birnbaum, L., Bois, F., Cogliano, V.J., Crofton, K., Euling, S.Y., Foster, P.M., Germolec, D.R., Gray, E., Hattis, D.B., Kyle, A.D., Luebke, R.W., Luster, M.I., Portier, C., Rice, D.C., Solomon, G., Vandenberg, J. and Zoeller, R.T. (2008): Meeting report: moving upstream-evaluating adverse upstream end points for improved risk assessment and decision-making. Environ. Health Perspect., **116**, 1568-1575.

Wullenweber, A., Kroner, O., Kohrman, M., Maier, A., Dourson, M., Rak, A., Wexler, P. and Tomljanovic, C. (2008): Resources for global risk assessment: the International Toxicity Estimates for Risk (ITER) and Risk Information Exchange (RiskIE) databases. Toxicol. Appl. Pharmacol., **233**, 45-53.

Xu, Y., Olman, V. and Xu, D. (2001): Minimum spanning trees for gene expression data clustering. Genome Inform., **12**, 24-33.

Xu, Y., Olman, V. and Xu, D. (2002): Clustering gene expression data using a graph-theoretic approach: an application of minimum spanning trees. Bioinformatics, **18**, 536-545.

BIOCHEMICAL PHARMACOLOGY 77 (2009) 660–669

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Review

Dioxin-induced toxicity on vascular remodeling of the placenta

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ARTICLE INFO

Article history:
Received 31 July 2008
Accepted 15 October 2008

Keywords:
Angiogenesis
Arylhydrocarbon receptor
Hypoxia
Placenta
Vasculogenesis

ABSTRACT

Arylhydrocarbon receptor (AhR) activated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) triggers its downstream signaling pathway to exert adverse effects on vasculature development, which can be initiated by vasculogenesis, followed by angiogenesis, or vascular remodeling, in a variety of animals including avians, piscines and mammals. The placenta, a mammalian organ rich in vasculature, consists of endothelial and trophoblast cells of fetal origin, which proliferate and differentiate under hypoxic condition in the uterine horn. Our studies demonstrated that vascular remodeling occurs prominently in the placenta of the control Holtzman rat strain during the late period of gestation, and induces changes in cell shape and elimination by apoptosis of trophoblasts. As a result, the net volumes of both maternal and fetal blood in the placenta increase to cope with the essential requirements of oxygen and nutrients in the late period of gestation. On the other hand, *in utero* exposure to TCDD markedly suppressed the development of sinusoids and trophoblast cells and the apoptosis of trophoblast cells with a concomitant increase in the incidence of fetal death under hypoxic condition. A crosstalk between the hypoxia-inducible factor (HIF)-mediated pathway and AhR-mediated pathway is considered to play an important role in this physiological process. No such changes were observed in the Sprague–Dawley rat strain that turned out to have an AhR conformation identical to that of the Holtzman rat strain. In this commentary, we will discuss a possible link of the TCDD toxicities with the AhR signaling pathway and gestation-related diseases.

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Abbreviations: AhR, arylhydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; GD, gestation day; HIF, hypoxia-inducible factor; HUVECs, human umbilical vein endothelial cells; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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doi:10.1016/j.bcp.2008.10.030

Contents

1. Vascular development and dioxin toxicity

Dioxin and related compounds, which belong to a family of halogenated aryl hydrocarbons, are produced unintentionally in uncontrolled combustion processes and in various types of industrial processes [1]. Among more than 400 kinds of congeners depending on the number and position of chlorine atoms on the benzene ring, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been established as the most toxic congener on the basis of experimental studies. The fetus is one of the most sensitive targets in the life of mammals, and *in utero* and lactational exposure to dioxins has been reported to elicit a wide spectrum of biological and toxicological responses, including reproductive, neurobehavioral, and immune disturbances in the offspring, by which dams are not affected as much as their fetuses [2]. Among the wide spectrum of toxicities, vascular development is particularly sensitive to the toxic effects of TCDD compared with congenital malformations or birth defects. Previous studies demonstrated that exposure to TCDD during development results in heart size reduction in the piscine embryo [3,4], dilatation of ventricular cavity associated with thinner ventricle walls in the chicken embryo [5] and decrease in heart-to-body weight in mice [6], all of which were accompanied by the reduction in cardiomyocyte proliferation. Edema and hemorrhage were observed and considered as common features of TCDD toxicities in the vasculature irrespective of animal species. Exposure to TCDD *in utero* induced subcutaneous edema and intestinal hemorrhages in the fetuses of the rat and hamster [7], and resulted in leakage from the vasculature in the morbid avian and piscine embryos, the latter of which was confirmed by severe subcutaneous, pericardial, and peritoneal edema prior to death [5,8]. TCDD clearly has a strong impact on living organisms by causing damage to the vascular system.

The toxicity of TCDD is mediated by the binding of TCDD to the arylhydrocarbon receptor (AhR), which is then activated. The activated ligand-bound AhR translocates to the nucleus from the cytoplasm and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT), followed by the binding of this AhR/ARNT heterodimer to the xenobiotic response element (XRE; also known as the dioxin response element, DRE) in the promoter region of a various genes [9]. If the XRE elements are functional, the AhR/ARNT heterodimer modulates the expression of those genes, including drug-metabolizing enzymes, i.e., phase I enzymes such as CYP1A1 and 1B1 and phase II enzymes such as UDP-glucuronosyl transferase, and biological and toxicological responses will emerge.

The vascular network plays a critical role at the very beginning of embryo development in order to supply oxygen and nutrients to adjacent proliferating or differentiated cells. Vasculature development in most of the organs is relatively simple and depends on two consecutive processes, vasculogenesis and angiogenesis [10] (Fig. 1A). In vasculogenesis, blood vessels form through the *in situ* differentiation between undifferentiated precursor cells, called angioblasts, to endothelial cells that assemble into a primitive vascular network, in which the adhesion of endothelial cells and periendothelial support cells is at the immature stage. The term angiogenesis was used to generally denote the growth and remodeling of the primitive network into a complex network. During this remodeling, periendothelial support cells are recruited to encase endothelial tubes, resulting in the maturation of blood vessels. In addition, some preexisting vessels send out capillary sprouts to produce new vessels. At each of these stages, growth factors and their receptors have been identified to act as modulators [10]. The vascular endothelial growth factor (VEGF) and its receptors (VEGFRs), such as fetal liver kinase-1 (Flk1) and fms-like tyrosine kinase-1 (Flt1), named the VEGF/VEGFR system, are mainly associated with vasculogenesis. Angiopoietin-1 (Ang1) and Ang2 and their receptor Tie2, named the Ang/Tie2 system, are involved mainly in angiogenesis, or vascular remodeling. Vascular development is basically stimulated under a hypoxic condition, which is dependent on transcription factors known as the hypoxia-inducible factors (HIFs). Under normal oxygen tension, HIF-1α is posttranslationally modified and subsequently degraded through the proteasome. However, under hypoxic conditions, HIF-1α can escape from degradation, and the accumulated HIF-1α binds to the oxygen-insensitive molecule known as the ARNT, also called HIF-1β. The HIF-1α/ARNT heterodimer subsequently binds to the hypoxia response element (HRE) in the promoter region of genes involved in the adaptation to hypoxia. Thus, the HIF-1α acts as a master regulator to activate the transcription of many hypoxia-response genes, including the VEGF/VEGFR or Ang/Tie2 system, and regulates the expressions of VEGF, Flt1, and Ang2 [11].

HIF-1α plays its intrinsic role in hypoxia signaling, and presumably modulates dioxin toxicities because of its ability to heterodimerize with ARNT [11]. In other words, ARNT is a common transcription factor that shares its role with AhR and HIF-1α to modulate XRE- and HRE-dependent pathways, respectively. Thus, it has been speculated that the AhR/ARNT and HIF-1α/ARNT pathways affect each other by competing for the limited quantities of ARNT molecules [12] (Fig. 1B).

Although it is considered that ARNT does not act as a limiting factor for the interaction with either AhR or HIF-1α because ARNT is abundant in cells under basal physiological condition [13], some of the experimental observations might be explained by the former hypothesis. In a study by Ichihara et al. [14], hypoxia caused by the ligation of the femoral artery was found to induce angiogenesis more significantly in AhR-null mice than in wild-type mice. In this study, electrophoretic mobility shift assay (EMSA) analysis showed that the DNA binding activity of the HIF-1α and ARNT complex is more pronounced in the AhR-null mice than in the wild-type mice under ischemic conditions. Thus, the authors suggest that the increased quantity and activity of the HIF-1α/ARNT heterodimer in ischemia-induced AhR-null mice may explain at least in part the enhancement of ischemia-induced VEGF expression and angiogenesis. In a study by Fritz et al. [15], transgenic adenocarcinoma of the mouse prostate (TRAMP) mice having AhR-null mutation develop prostate tumors with greater frequency than AhR-positive TRAMP mice. The authors showed that the stimulated development of the prostate tumor in AhR-null TRAMP mice is due to the accelerated angiogenesis resulting from the increased VEGF expression on the prostate epithelial hyperplasia, a typical malformation observed in TRAMP mice. Because the addition of vanadate, a putative inducer of the HIF-1α-VEGF pathway, resulted in VEGF induction in the organ culture experiment of the prostate obtained from AhR-null mice but not from WT mice, the authors concluded that the increased VEGF production in AhR-null TRAMP mice is due to the overstimulated HIF-1α/ARNT signaling pathway. On the other hand, exposure to TCDD or 3-methylcholanthrene decreased the VEGF expression under several experimental conditions such as in the case of coronary endothelial tube formation in chick embryos [16–18] and human umbilical vein endothelial cells (HUVECs) *in vitro* [19]. Exposure to cigarette smoke extract was shown to suppress the hypoxia-induced cellular migration and capillary-like tubule formation in HUVECs *in vitro*. In an *in vivo* experiment, blood flow perfusion in surgically induced ischemic hind limbs was significantly reduced in mice exposed to cigarette smoke. In these *in vitro* and *in vivo* experiments, the expression of HIF-1α/VEGF was downregulated [20]. These observations suggest that the AhR-dependent reduction of VEGF expression by TCDD and other AhR ligands may result in a tilted balance toward the AhR/ARNT pathway instead of the HIF-1α/ARNT pathway. It should be noted that the term 'angiogenesis' has often been used to include the

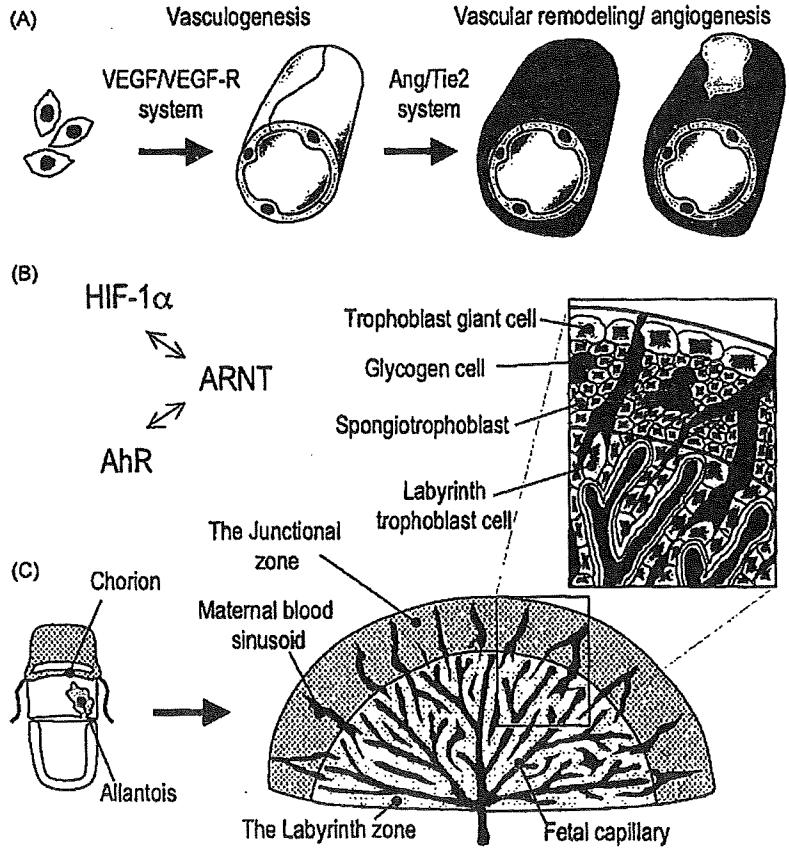

Fig. 1 – Vascular development and structure in the peripheral blood vessels and placenta. (A) In the peripheral blood vessels, a primitive vascular tube is formed during vasculogenesis, which is further processed by remodeling its structure to recruit peripheral endothelial support cells and by sending out capillary sprouts to produce new vessels. (B) Proposed model for suppressive effects of AhR-mediated signaling on HIF-1α signaling pathway by competing for limited amounts of ARNT. (C) Development of the rodent placenta. Placental development is initiated by fusing two membranes, the chorion and allantois. The mature rodent placenta is composed of the labyrinth and junctional zones as described in the text.

term 'vasculogenesis' in a number of papers, and thus, one has to pay particular attention to the blood vessel development stage described in a given paper. Generally, it is plausible to think that not only the vascular remodeling but also vasculogenesis is considered to be a target of AhR ligands.

Although it is still controversial, competition of AhR and HIF-1α with ARNT is a plausible model to work on to elucidate the inhibitory mechanisms of the AhR-mediated signaling pathway on HIF-1α signaling activity. Intriguingly, Ohtake et al. recently found a novel function of AhR [21,22]. The AhR has E3 ubiquitin-ligase activity by forming multiple protein complexes and degraded several transcription factors including the estrogen and androgen receptors. The substrates for AhR-mediated ubiquitin ligase have not been fully identified, and thus, whether HIF-1α is a target of AhR-mediated ubiquitin-ligase activity is yet unknown. In either case, the inhibitory effect of the AhR-mediated signaling pathway on the HIF-1α signaling pathway is due to the downregulation of active HIF-1α, which is consistent with the results of *in vivo* studies. Another possibility of the inhibitory effect of HIF-1α on AhR-mediated gene transcription is a competition of transcription cofactors between these nuclear receptors. Several nuclear receptor coactivators are known to interact with the AhR, including ERAP140 [23], RIP140 [24], CBP/P300 [25], BRG-1 [26], and the three members of the p160 family of coactivators: NCoA1 (SRC-1), NCoA2 (GRIP-1 and TIF-2) and NCoA3 (AIB-1, p/CIP, and ACTR) [27]. On the other hand, HIF-1α is known to interact with CBP/p300, SRC-1 and TIF2 [28] [29–32]. Thus, it is plausible that AhR and HIF-1α competes for a limited amount of CBP/p300, which may suppress transcriptional activities of these nuclear receptors. For further study, it is necessary to clarify the exact inhibitory mechanism of the AhR-mediated signaling pathway on the HIF-1α signaling pathway.

2. Vascular development in the placenta and related diseases

The placenta is an organ penetrated by maternal and fetal blood vessels, and acts as an interface between them by exchanging oxygen, nutrients and by-products. The vasculature of the placenta has been extensively studied because of its central role in pathogenesis for both maternal and fetal sides. Among mammalian species, the anatomical structures of rodents and humans are similar. The mature rodent placenta, so-called chorioallantoic placenta, is morphologically divided into two zones, the labyrinth zone and the junctional zone [33] (Fig. 1C). The junctional zone, which is devoid of fetal blood, contains three types of cells of fetal origin, i.e., the spongiotrophoblast cells, glycogen cells and trophoblast giant cells. Glycogen cells are considered to supply energy while spongiotrophoblast cells and trophoblast giant cells are known to secrete hormones, including diverse types of placental prolactin family proteins in a stage-specific manner [34]. The labyrinth zone is a place for exchanging oxygen and nutrients between maternal and fetal blood.

The most striking event during the development of the placental vasculature is the fusion of two membranes, chorion and allantois, and this event, termed as chorioallantoic fusion, begins around gestational day (GD) 10 and GD8.5 in rat and mouse, respectively [35] (Fig. 1C). After this fusion, fetal capillaries grow from the allantois, and the trophoblast cells that mostly originated from the chorion undergo extensive villous branching with its associated fetal capillaries to construct a vasculature in the labyrinth. Around the period of chorioallantoic fusion, the trophoblast giant cells invade into the maternal uterine wall, and maternal blood that leaks from ruptured uterine vessels flows into the narrow space of the labyrinthine maternal blood sinusoids that directly contact with the labyrinthine trophoblast cells. The labyrinthine vasculature is estimated to develop until around GD15 in the rat when DNA synthesis is terminated thereafter [36,37]. A crucial point raised here is that two different types of cell, endothelial and trophoblast cells, actively participate in the establishment of the complex vasculature of the placenta, whereas only endothelial cells play a major role in the development of the vasculature in other organs.

Even though vascular development in the placenta is more complicated than in other organs, knockout mouse studies showed that vascular development is strictly regulated by VEGF and HIFs in the placenta, which is similar to that in other organs. It was reported that embryos deficient in HIF-1α or ARNT are viable up to GD9.5 but could not survive beyond GD10.5, owing to severe placental defects including shallow placental invasion into the decidua and lack of vascularization of fetal vessels in the labyrinth zone because of a defect in the chorioallantoic fusion [38–41]. Furthermore, the number of spongiotrophoblast cells in the junctional zone was markedly reduced, whereas that of trophoblasts cells in the labyrinth zone was increased, suggesting that the balance of trophoblastic differentiation into each lineage was tilted [41]. These results strongly suggest that the differentiation of trophoblast cells is strictly regulated by HIF-1α/ARNT.

Impairment of placental blood circulation often results in disease conditions, such as intrauterine growth retardation of the fetus and preeclampsia, the latter of which is characterized by hypertension and proteinuria in pregnant women [42,43]. Approximately 5–7% of all pregnant women develop preeclampsia. Although the precise etiology is not known, preeclampsia is accompanied by vasospasm and endothelial injury as an end result [44]. Excessive secretion of Flt1 is considered to be responsible for endothelial injury [45]. Both disease conditions are presumably related to each other, and preeclampsia sometimes accompanies intrauterine growth restriction. In temporal aspects, these symptoms manifest during the late period of gestation, suggesting that vascular remodeling might participate in the development of this disease. Recent studies reported a possible link of preeclampsia of humans with that of rodents [35,46].

The etiologies of these diseases are complex owing to several factors including genetic as well as environmental issues [47]. As an environmental factor, cigarette smoking has been reported to impair placental vasculature and subsequent fetal growth restriction [48,49]. Microarray analysis, followed by quantitative RT-PCR analysis, of gene expression in the placentas of cigarette-smoking mothers revealed that AhR-dependent phase I enzyme genes, such as cytochrome P450 1A1 (CYP1A1) and CYP1B1, are activated, but that AhR- or Nrf2-dependent phase II genes are not. The imbalance between the

induced phase I enzymes and the noninduced phase II enzymes may result in increased oxidative stress, which could interfere with the function of the placenta and adversely affect the well-being of the fetus [50]. It is thus plausible to consider that AhR ligands act as environmental factors that affect the normal development of the placental vasculature.

3. Effects of TCDD on vascular remodeling in the placenta and the proposed mechanisms of toxicities

Because AhR ligands including dioxins and related compounds affect the early stage of organ development, it is intriguing to study how AhR-mediated signaling is involved in the development of blood vessels in the placenta. Administration of TCDD to pregnant C57BL/6 mice at a daily dose of 3 or 6 μg/kg bw from GDs 10 to 13 was found to induce histological alterations 24 h after the last administration when vasculogenesis is supposed to continue [51]. In the TCDD-exposed placenta, the vasculature that acts as a maternal-fetal barrier in the labyrinth was found to show hemorrhage of embryonic blood into the maternal circulation. This data suggests that the dose used was too high to consider subtle changes in terms of the expression of molecular markers of vasculogenesis and vascular remodeling. It has been reported that the exposure of Holtzman, Long–Evans, or Sprague–Dawley rats to TCDD before fertilization or at the early stage of gestation results in fetal death at the late, but not early, stage of gestation [7,52–54]. In these studies, no detailed analysis of the histology and molecular markers was available, and it is difficult to conclude whether and how TCDD affects chorioallantoic fusion, placental vasculogenesis, and vascular remodeling.

In the control placenta of Holtzman rats, the vascular remodeling were found to take place on GD15 even when the placental DNA synthesis was already terminated and, therefore, the vasculogenesis in the placenta presumably ceased [55]. On GD16, maternal sinusoids and fetal capillaries were narrow and the size and thickness of trophoblast cells were small, but these morphological features became reverse on GD20 with the development of the placenta (Fig. 2). In addition, the upregulation of genes involved in both the VEGF/VEGFR and Ang/Tie2 systems during this period was observed. On the other hand, administration of TCDD at 1600 ng/kg bw to Holtzman rats on GD15 to study its possible effects on the placental vasculature in the late period of gestation, the morphological features, such as maternal sinusoid, fetal capillaries and trophoblast cells, of the TCDD-exposed placenta on GD20, were very similar to the ones of the control placenta on GD16 (Fig. 2B) [55]. Lack of dilatation of both maternal blood sinusoids and fetal capillaries, existence of large size trophoblast cells, and the downregulated Tie2 mRNA level among the VEGF/VEGFR and Ang/Tie2 systems were

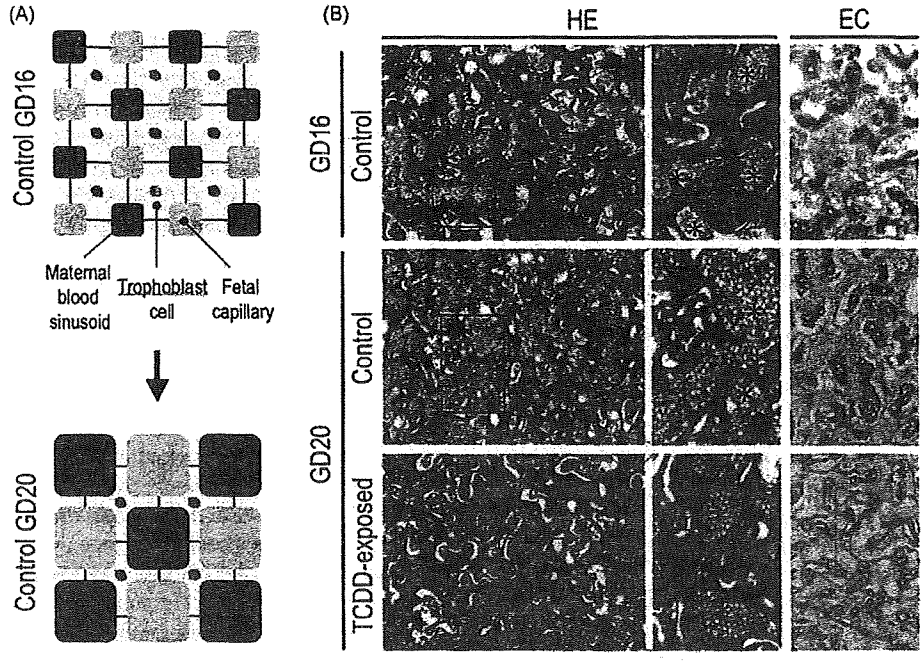

Fig. 2 – Vascular remodeling occurs in the placenta of Holtzman rats during the late period of gestation. (A) Schematic of horizontal dimension in the labyrinth zone of the placenta. Both the maternal blood sinusoids and fetal capillaries are enlarged from GDs 16 to 20 with a concomitant decrease in the number as well as thinning of trophoblast cells. (B) Suppresstion of vascular remodeling in the TCDD-exposed placenta. Horizontal sections of the placentas on GD16 and GD20 are shown. These sections were subjected to either hematoxylin and eosin (HE) staining, or endothelial cell (EC) staining using BS-1 lectin that identifies fetal capillaries. Note that the maternal blood sinusoids (asterisks) and fetal capillaries (in the EC stain) are not expanded in the TCDD-exposed placenta on GD20, which is similar to the morphology of control placenta on GD16. In addition, the number (arrow heads) and size (circle) of trophoblast cells are not decreased, and the trophoblastic interhemal membrane (between arrows) does not become thin in the TCDD-exposed placenta on GD20.

observed in TCDD-exposed placentas on GD20, suggesting that the vascular remodeling was suppressed by TCDD exposure. Furthermore, a striking effect of TCDD on apoptosis of trophoblast cells was present. Similar to the human placenta during the late period of gestation [56], a significant number of trophoblast cells (approximately 500/mm^2) were dead by apoptosis in the control placenta from GDs 16 to 20. In contrast, the number of apoptotic trophoblast cells in the TCDD-exposed placenta decreased to less than half of that in the control placenta [55]. In addition, TCDD-exposed placentas on GD20 were found to be in hypoxic condition [57], and to have an altered glucose metabolism on GD20 [58]. Under this condition, the presence of the altered glucose and glycogen metabolism was supported by the observations of decomposition products of glycogen cells in the junctional zone, increased glycogen content and upregulation of glucose transporter-2 (GLUT-2), GLUT-3, and GLUT-4 mRNAs levels [58,59].

The vascular remodeling to expand spaces for maternal blood sinusoids as well as fetal capillaries, observed in the control placentas during the late period of gestation, is considered to increase the net volume of circulating blood within the placenta. The observation is congruent with the increase in the physiological need for oxygen and nutrients of the significantly growing fetus. The apoptosis of tropohoblast cells is considered indispensable to offer spaces for the expansion of maternal blood sinusoids as well as fetal capillaries because the size of the placenta is restricted within the uterine horn and is relatively constant during the late period of gestation. Therefore, the inhibition of apoptosis by TCDD is considered to be essential for the pathogenesis of the TCDD-suppressive effect on vascular remodeling in the placenta of Holtzman rats.

The suppression of apoptosis in other types of cells following TCDD exposure has been reported in other studies. Vogel et al. [60] showed that the activation of AhR by TCDD resulted in the loss of the apoptosis response in lymphoma cell lines, which plays a key role in the development of lymphoma and leukemia, and clarified that the upregulation of cyclooxygenase-2 (COX-2), a downstream gene in the AhR signaling pathway, is associated with the suppression of apoptosis. Ray and Swanson [61] showed that TCDD exposure induces immortalization of human keratinocytes by suppressing apoptosis. Stinchcombe et al. [62] showed that the tumor promotion activated by TCDD in the rat liver is due to a decrease in apoptotic level. In this regard, the apoptotic activity of the human mammary epithelial cell line MCF10A was suppressed by TCDD exposure [63,64]. These several lines of evidence suggest that the suppression of apoptosis is considered to be a fundamental to TCDD toxicities.

How this inhibitory effect of TCDD on apoptosis is regulated? Paajarvi et al. [65] suggested that mouse double minute 2 (MDM2) which is up-regulated by AhR-mediated pathway is involved in this process. They showed that pretreatment of rats with TCDD diminished diethylnitrosamine-induced apoptosis in liver cells that is known to be p53-dependent, and that up-regulated MDM2 by AhR-mediated pathway decreased the apoptosis because MDM2 binds and degrades p53. It was reported that upregulation of MDM2 by nuclear receptor CAR is critical to suppress apoptosis in liver of mice that are exposed to pesticide contaminant 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) [66]. These results suggest that the MDM2 might play a central role in suppressing apoptosis after exposure to xenobiotics including TCDD. Further studies are necessary to assess whether MDM2 is upregulated in the placenta, especially in the trophoblast cells, after exposure to TCDD.

Chorioallantoic fusion and subsequent vasculogenesis have been proved to be regulated by the HIF-1α/ARNT signaling pathway [38–41]. The HIF-1α/ARNT pathway is possibly activated under hypoxic condition in the late period of gestation owing to an increased demand for oxygen during the fetal growth. This activation then stimulates the vascular remodeling, which results in an increase in the blood supply to overcome the hypoxic condition. In reality, these responses are highly tuned to be in a dynamic equilibrium. Our proteomics analysis data suggests that TCDD-induced placental tissues are under hypoxic status, and that the above-mentioned equilibrium is disrupted owing to the suppression of vascular remodeling in the placenta [55]. Trophoblast cells have a unique HIF-1α/ARNT signaling pathway compared with cells in other organs in order to thrive in the innate low-oxygen environment. This notion is supported by the experimental evidence showing that knockout mice of either ARNT or von Hippel-Lindau (VHL) gene, the product of which interacts with HIF-1α, has defect of blood vessel formation only in the placenta but not in yolk sac and embryos [67,68]. Further studies are required to clarify how the HIF-1α/ARNT signaling pathway regulates vascular remodeling in the placenta.

Type 1 diabetic mouse model that is induced by administration of streptozotocin exhibits intrauterine growth retardation [69]. Intriguingly, the placentas of these mice exhibit similar to those in TCDD-exposed rats including reduced blood flow [69], containing decomposition products of glycogen cells in the junctional zone [70,71], increased glycogen content [72,73], and increased GLUT3 mRNA level [74]. Moreover, fetuses in cadmium-exposed pregnant rats [75] and ethanol-fed pregnant mice [76] exhibit intrauterine growth retardation concomitant with the appearance of decomposition products of glycogen cells in the placenta. Therefore, it is conceivable that the placenta exhibits prototypical symptoms including abnormal vasculature and altered glucose kinetics regardless of the different types of insult as above. All of these insults, as well as smoking, could induce intrauterine growth retardation by disrupting the proper function of the placental vasculature in not only rodents but also humans [77]. A common process underlying the onset of these disease models is production of excessive amounts of reactive oxygen species that may cause damage to tissues, which has also been observed in TCDD-exposed placenta [76,77]. The suppression by TCDD of vascular remodeling in the placenta should be addressed on the basis of two important aspects, the involvement of the HIF-1a/ARNT pathway and the causal relationship between vascular impairment and oxidative stress.

4. Susceptibility of the fetus to TCDD toxicity and the AhR structure

Because the growing fetus requires large quantities of oxygen and nutrients particularly during the late period of gestation, a

failure in vascular remodeling is considered to increase the risk of fetal death. When Holtzman rats were administered TCDD at a dose of 0.8 and 1.6 μg/kg bw on GD15, they developed a disorder in the vasculature of the labyrinth zone, and the incidences of fetal death were 1% and 13%, respectively [58]. On the other hand, a low susceptibility of Sprague–Dawley rats to an *in utero* exposure to TCDD was found in an experiment, that is, even a six fold higher dose of TCDD at 10 μg/kg bw failed to cause any pathological alterations in the morphology of the placental vasculature and fetal death [78]. A logical explanation would be that the primary structures of the AhRs of these two rat strains differ from each other, as has been established in mouse and rat strains [9,79–81]. For example, in mouse strains, the affinity of the AhR of the C57BL/6J strain to TCDD is much higher than that of the AhR of other strains, such as DBA/2 [82]. In rat strains, Long-Evans rats have AhR that has a higher affinity to TCDD and are at least 1000-fold more sensitive (LD_{50} about 10 μg/kg) to the acute lethal effects of TCDD than Han/Wistar (kuopio; H/W) rats [83]. We thus formulated the above-mentioned hypothesis and examined the sequence of AhR. Contradictory to our hypothesis, the primary structure of AhR was found to be identical between Holtzman and Sprague–Dawley rats. We next examined the possible differences in the activity of AhR between these two rats strains by examining the TCDD-dependent expression of CYP1A1 mRNA, and confirmed that both rat strains induced CYP1A1 mRNA at an identical level in the placentas [78], suggesting that the strain difference in the TCDD toxicities on the placental vasculature and fetal death does not depend on the magnitude of AhR activities during gene transcription. Although we did not determine TCDD concentrations in the placenta, identical CYP1A1 mRNA levels suggest that TCDD was retained in this tissue presumably at a similar level.

To study the possible relationship of placental abnormalities with fetal death, we compared how the vascular structures of Sprague–Dawley and Holtzman rats develop as the gestation proceeds. In normal Sprague–Dawley rats, the vasculature in the labyrinth zone on GD16 is immature as shown by the narrow shape of both maternal blood sinusoids and fetal capillaries (unpublished data), and such morphology is very similar to that of Holtzman rats on GD16. However, the vasculature in the labyrinth zone of Sprague–Dawley rats did not change even on GD20. That is, the trophoblast cells are still large and their interhemal membrane is also thick in the labyrinth. In contrast, the vasculature in the labyrinth zone in normal Holtzman rats is altered from GDs 16 to 20, as described above. It seems likely that apoptotic elimination of trophoblast cells is decreased, or even does not occur in the placenta of Sprague–Dawley rats, suggesting that placental vascular development under the control of VEGF/VEGF-R and/or Ang/Tie2 systems of this strain might be different from that of Holtzman rats. The comparative analysis of the expression of molecular markers such as VEGF/VEGF-R and Ang/Tie2 systems during gestation could possibly unravel the mechanisms underlying vascular development and TCDD toxicity.

5. Conclusions

Vascular development, initiated by vasculogenesis angiogenesis (vascular remodeling), is regulated by an orchestration of VEGF/VEGF-R and Ang/Tie2 systems, respectively. In particular, the VEGF/VEGF-R system is upregulated by HIF-1α/ARNT under hypoxic condition in vasculogenesis, leading to the activation of the Ang/Tie2 system in vascular remodeling. It was demonstrated that *in utero* exposure to TCDD or cigarette smoke that contains AhR ligands affects vasculogenesis and vascular remodeling via AhR signaling by interacting with HIF-1α signaling depending on the vascular development stage. We found that *in utero* exposure to TCDD affects the process of vascular remodeling rather than vasculogenesis in the placenta of Holtzman rats. In this study, it was found that *in utero* exposure to TCDD markedly suppressed the development of sinusoids and trophoblast cells and the apoptosis of trophoblast cells under hypoxic condition, which results in a higher incidence of fetal death. However, no such effects were observed in Sprague–Dawley rats even if these two rat strains had identical AhR structure. The elucidation of the physiological process of vascular remodeling in these rat strains may shed light on how AhR signaling is involved in the TCDD toxicities in the placental vasculature.

Acknowledgements

This research was supported in part by the Special Coordination Fund for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology (to R.I.) and CREST, JST (to C.T.).

REFERENCES

[1] Hutzinger O, Choudhry GG, Chittim BG, Johnston LE. Formation of polychlorinated dibenzofurans and dioxins during combustion, electrical equipment fires and PCB incineration. Environ Health Perspect 1985;60:3–9.
[2] Theobald HM, Kimmel GL, Peterson RE. Developmental and reproductive toxicity of dioxins and related chemicals. In: Schecter A, Gasiewicz TA, editors. Dioxins and health. Hoboken, NJ, USA: Wiley-Interscience; 2003 . p. 329–432.
[3] Antkiewicz DS, Burns CG, Carney SA, Peterson RE, Heideman W. Heart malformation is an early response to TCDD in embryonic zebrafish. Toxicol Sci 2005;84:368–77.
[4] Antkiewicz DS, Peterson RE, Heideman W. Blocking expression of AHR2 and ARNT1 in zebrafish larvae protects against cardiac toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Sci 2006;94:175–82.
[5] Walker MK, Catron TF. Characterization of cardiotoxicity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related chemicals during early chick embryo development. Toxicol Appl Pharmacol 2000;167:210–21.
[6] Thackaberry EA, Nunez BA, Ivnitski-Steele ID, Friggins M, Walker MK. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on murine heart development: alteration in fetal and postnatal cardiac growth, and postnatal cardiac chronotropy. Toxicol Sci 2005;88:242–9.
[7] Olson JR, Holscher MA, Neal RA. Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the golden Syrian hamster. Toxicol Appl Pharmacol 1980;55:67–78.
[8] Guiney PD, Walker MK, Spitsbergen JM, Peterson RE. Hemodynamic dysfunction and cytochrome P4501A mRNA expression induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin

during embryonic stages of lake trout development. Toxicol Appl Pharmacol 2000;168:1–14.
[9] Mimura J, Fujii-Kuriyama Y. Functional role of AhR in the expression of toxic effects by TCDD. Biochim Biophys Acta 2003;1619:263–8.
[10] Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000;6:389–95.
[11] Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–32.
[12] Nie M, Blankenship AL, Giesy JP. Interactions between aryl hydrocarbon receptor (AhR) and hypoxia signaling pathways. Environ Toxicol Pharmacol 2001;10:17–27.
[13] Pollenz RS, Davarinos NA, Shearer TP. Analysis of aryl hydrocarbon receptor-mediated signaling during physiological hypoxia reveals lack of competition for the aryl hydrocarbon nuclear translocator transcription factor. Mol Pharmacol 1999;56:1127–37.
[14] Ichihara S, Yamada Y, Ichihara G, Nakajima T, Li P, Kondo T, et al. A role for the aryl hydrocarbon receptor in regulation of ischemia-induced angiogenesis. Arterioscler Thromb Vasc Biol 2007;27:1297–304.
[15] Fritz WA, Lin TM, Peterson RE. The aryl hydrocarbon receptor (AhR) inhibits vanadate-induced vascular endothelial growth factor (VEGF) production in TRAMP prostates. Carcinogenesis 2008;29:1077–82.
[16] Ivnitski-Steele ID, Walker MK. Vascular endothelial growth factor rescues 2,3,7,8-tetrachlorodibenzo-p-dioxin inhibition of coronary vasculogenesis. Birth Defects Res A Clin Mol Teratol 2003;67:496–503.
[17] Ivnitski-Steele ID, Sanchez A, Walker MK. 2,3,7,8-Tetrachlorodibenzo-p-dioxin reduces myocardial hypoxia and vascular endothelial growth factor expression during chick embryo development. Birth Defects Res A Clin Mol Teratol 2004;70:51–8.
[18] Ivnitski-Steele ID, Friggens M, Chavez M, Walker MK. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) inhibition of coronary vasculogenesis is mediated, in part, by reduced responsiveness to endogenous angiogenic stimuli, including vascular endothelial growth factor A (VEGF-A). Birth Defects Res A Clin Mol Teratol 2005;73:440–6.
[19] Juan SH, Lee JL, Ho PY, Lee YH, Lee WS. Antiproliferative and antiangiogenic effects of 3-methylcholanthrene, an aryl-hydrocarbon receptor agonist, in human umbilical vascular endothelial cells. Eur J Pharmacol 2006;530:1–8.
[20] Michaud SE, Menard C, Guy LG, Gennaro G, Rivard A. Inhibition of hypoxia-induced angiogenesis by cigarette smoke exposure: impairment of the HIF-1alpha/VEGF pathway. FASEB J 2003;17:1150–2.
[21] Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, et al. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature 2003;423:545–50.
[22] Ohtake F, Baba A, Takada I, Okada M, Iwasaki K, Miki H, et al. Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. Nature 2007;446:562–6.
[23] Nguyen TA, Hoivik D, Lee JE, Safe S. Interactions of nuclear receptor coactivator/corepressor proteins with the aryl hydrocarbon receptor complex. Arch Biochem Biophys 1999;367:250–7.
[24] Kumar MB, Tarpey RW, Perdew GH. Differential recruitment of coactivator RIP140 by Ah and estrogen receptors. Absence of a role for LXXLL motifs. J Biol Chem 1999;274:22155–64.
[25] Marlowe JL, Knudsen ES, Schwemberger S, Puga A. The aryl hydrocarbon receptor displaces p300 from E2F-dependent promoters and represses S phase-specific gene expression. J Biol Chem 2004;279:29013–22.
[26] Wang S, Hankinson O. Functional involvement of the Brahma/SWI2-related gene 1 protein in cytochrome P4501A1 transcription mediated by the aryl hydrocarbon receptor complex. J Biol Chem 2002;277:11821–7.
[27] Beischlag TV, Wang S, Rose DW, Torchia J, Reisz-Porszasz S, Muhammad K, et al. Recruitment of the NCoA/SRC-1/p160 family of transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator complex. Mol Cell Biol 2002;22:4319–33.
[28] Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, et al. An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci USA 1996;93:12969–73.
[29] Ebert BL, Bunn HF. Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor, and p300/CREB binding protein. Mol Cell Biol 1998;18:4089–96.
[30] Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H, et al. Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. EMBO J 1998;17:6573–86.
[31] Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, et al. Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J 1999;18:1905–14.
[32] Carrero P, Okamoto K, Coumailleau P, O'Brien S, Tanaka H, Poellinger L. Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. Mol Cell Biol 2000;20:402–15.
[33] Davies J, Glasser SR. Histological and fine structural observations on the placenta of the rat. Acta Anat (Basel) 1968;69:542–608.
[34] Soares MJ. The prolactin and growth hormone families: pregnancy-specific hormones/cytokines at the maternal–fetal interface. Reprod Biol Endocrinol 2004;2:51.
[35] Rossant J, Cross JC. Placental development: lessons from mouse mutants. Nat Rev Genet 2001;2:538–48.
[36] Butterstein GM, Leathem JH. Placental growth modification during pregnancy in the rat. Endocrinology 1974;95:645–9.
[37] Winick M, Noble A. Quantitative changes in ribonucleic acids and protein during normal growth of rat placenta. Nature 1966;212:34–5.
[38] Adelman DM, Gertsenstein M, Nagy A, Simon MC, Maltepe E. Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes Dev 2000;14:3191–203.
[39] Kozak KR, Abbott B, Hankinson O. ARNT-deficient mice and placental differentiation. Dev Biol 1997;191:297–305.
[40] Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature 1997;386:403–7.
[41] Cowden Dahl KD, Fryer BH, Mack FA, Compernolle V, Maltepe E, Adelman DM, et al. Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Mol Cell Biol 2005;25:10479–91.
[42] Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science 2005;308:1592–4.
[43] Kaufmann P, Black S, Huppertz B. Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. Biol Reprod 2003;69:1–7.
[44] Barbieri RL, Repeke JT. Medical disorders during pregnancy. In: Braunwald E, Fauchi A, Kasper D, Hauser S, Longo D, Jameson J, editors. Harrison's Principles Internal Medicine. 16th ed., McGraw-Hill; 2005. 32–37.
[45] Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension,

and proteinuria in preeclampsia. J Clin Invest 2003;111:649–58.
[46] Cross JC. The genetics of pre-eclampsia: a feto-placental or maternal problem? Clin Genet 2003;64:96–103.
[47] Ilekis JV, Reddy UM, Roberts JM. Preeclampsia—a pressing problem: an executive summary of a National Institute of Child Health and Human Development workshop. Reprod Sci 2007;14:508–23.
[48] Salafia C, Shiverick K. Cigarette smoking and pregnancy II: vascular effects. Placenta 1999;20:273–9.
[49] Zdravkovic T, Genbacev O, McMaster MT, Fisher SJ. The adverse effects of maternal smoking on the human placenta: a review. Placenta 2005;26 Suppl. A:S81–6.
[50] Huuskonen P, Storvik M, Reinisalo M, Honkakoski P, Rysa J, Hakkola J, et al. Microarray analysis of the global alterations in the gene expression in the placentas from cigarette-smoking mothers. Clin Pharmacol Ther 2008;83:542–50.
[51] Khera KS. Extraembryonic tissue changes induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin and 2,3,4,7,8-pentachlorodibenzofuran with a note on direction of maternal blood flow in the labyrinth of C57BL/6N mice. Teratology 1992;45:611–27.
[52] Sparschu GL, Dunn FL, Rowe VK. Study of the teratogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the rat. Food Cosmet Toxicol 1971;9:405–12.
[53] Murray FJ, Smith FA, Nitschke KD, Humiston CG, Kociba RJ, Schwetz BA. Three-generation reproduction study of rats given 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the diet. Toxicol Appl Pharmacol 1979;50:241–52.
[54] Huuskonen H, Unkila M, Pohjanvirta R, Tuomisto J. Developmental toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the most TCDD-resistant and -susceptible rat strains. Toxicol Appl Pharmacol 1994;124:174–80.
[55] Ishimura R, Kawakami T, Ohsako S, Nohara K, Tohyama C. Suppressive effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on vascular remodeling that takes place in the normal labyrinth zone of rat placenta during late gestation. Toxicol Sci 2006;91:265–74.
[56] Straszewski-Chavez SL, Abrahams VM, Mor G. The role of apoptosis in the regulation of trophoblast survival and differentiation during pregnancy. Endocr Rev 2005;26:877–97.
[57] Ishimura R, Ohsako S, Kawakami T, Sakaue M, Aoki Y, Tohyama C. Altered protein profile and possible hypoxia in the placenta of 2,3,7,8-tetrachlorodibenzo-p-dioxin-exposed rats. Toxicol Appl Pharmacol 2002;185:197–206.
[58] Ishimura R, Ohsako S, Miyabara Y, Sakaue M, Kawakami T, Aoki Y, et al. Increased glycogen content and glucose transporter 3 mRNA level in the placenta of Holtzman rats after exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Appl Pharmacol 2002;178:161–71.
[59] Mizutani T, Yoshino M, Satake T, Nakagawa M, Ishimura R, Tohyama C, et al. Identification of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible and -suppressive genes in the rat placenta: induction of interferon-regulated genes with possible inhibitory roles for angiogenesis in the placenta. Endocr J 2004;51:569–77.
[60] Vogel CF, Li W, Sciullo E, Newman J, Hammock B, Reader JR, et al. Pathogenesis of aryl hydrocarbon receptor-mediated development of lymphoma is associated with increased cyclooxygenase-2 expression. Am J Pathol 2007;171:1538–48.
[61] Ray SS, Swanson HI. Dioxin-induced immortalization of normal human keratinocytes and silencing of p53 and p16INK4a. J Biol Chem 2004;279:27187–93.
[62] Stinchcombe S, Buchmann A, Bock KW, Schwarz M. Inhibition of apoptosis during 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated tumour promotion in rat liver. Carcinogenesis 1995;16:1271–5.
[63] Park S, Matsumura F. Characterization of anti-apoptotic action of TCDD as a defensive cellular stress response reaction against the cell damaging action of ultra-violet irradiation in an immortalized normal human mammary epithelial cell line, MCF10A. Toxicology 2006;217:139–46.
[64] Davis Jr JW, Burdick AD, Lauer FT, Burchiel SW. The aryl hydrocarbon receptor antagonist, 3'methoxy-4'nitroflavone, attenuates 2,3,7,8-tetrachlorodibenzo-p-dioxin-dependent regulation of growth factor signaling and apoptosis in the MCF-10A cell line. Toxicol Appl Pharmacol 2003;188:42–9.
[65] Paajarvi G, Viluksela M, Pohjanvirta R, Stenius U, Hogberg J. TCDD activates Mdm2 and attenuates the p53 response to DNA damaging agents. Carcinogenesis 2005;26:201–8.
[66] Huang W, Zhang J, Washington M, Liu J, Parant JM, Lozano G, et al. Xenobiotic stress induces hepatomegaly and liver tumors via the nuclear receptor constitutive androstane receptor. Mol Endocrinol 2005;19:1646–53.
[67] Gnarra JR, Ward JM, Porter FD, Wagner JR, Devor DE, Grinberg A, et al. Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. Proc Natl Acad Sci USA 1997;94:9102–7.
[68] Abbott BD, Buckalew AR. Placental defects in ARNT-knockout conceptus correlate with localized decreases in VEGF-R2, Ang-1, and Tie-2. Dev Dyn 2000;219:526–38.
[69] Chartrel NC, Clabaut MT, Boismare FA, Schrub JC. Uteroplacental hemodynamic disturbances in establishment of fetal growth retardation in streptozocin-induced diabetic rats. Diabetes 1990;39:743–6.
[70] Prager R, Abramovici A, Liban E, Laron Z. Histopathological changes in the placenta of streptozotocin induced diabetic rats. Diabetologia 1974;10:89–91.
[71] Gewolb IH, Merdian W, Warshaw JB, Enders AC. Fine structural abnormalities of the placenta in diabetic rats. Diabetes 1986;35:1254–61.
[72] Abramovici A, Sporn J, Prager R, Shaltiel A, Laron Z, Liban E. Glycogen metabolism in the placenta of streptozotocin diabetic rats. Horm Metab Res 1978;10:195–9.
[73] Gewolb IH, Barrett C, Warshaw JB. Placental growth and glycogen metabolism in streptozotocin diabetic rats. Pediatr Res 1983;17:587–91.
[74] Boileau P, Mrejen C, Girard J, Hauguel-de Mouzon S. Overexpression of GLUT3 placental glucose transporter in diabetic rats. J Clin Invest 1995;96:309–17.
[75] Hazelhoff Roelfzema W, Roelofsen AM, Peereboom-Stegeman JH. Glycogen content of placenta and of fetal and maternal liver in cadmium-exposed rats. I. A descriptive light microscopic study. Placenta 1987;8:27–36.
[76] Padmanabhan R. Histological and histochemical changes of the placenta in fetal alcohol syndrome due to maternal administration of acute doses of ethanol in the mouse. Drug Alcohol Depend 1985;16:229–39.
[77] Maulik D. Fetal growth restriction: the etiology. Clin Obstet Gynecol 2006;49:228–35.
[78] Kawakami T, Ishimura R, Nohara K, Takeda K, Tohyama C, Ohsako S. Differential susceptibilities of Holtzman and Sprague–Dawley rats to fetal death and placental dysfunction induced by 2,3,7,8-teterachlorodibenzo-p-dioxin (TCDD) despite the identical primary structure of the aryl hydrocarbon receptor. Toxicol Appl Pharmacol 2006;212:224–36.
[79] Moriguchi T, Motohashi H, Hosoya T, Nakajima O, Takahashi S, Ohsako S, et al. Distinct response to dioxin in an arylhydrocarbon receptor (AHR)-humanized mouse. Proc Natl Acad Sci USA 2003;100:5652–7.
[80] Tuomisto JT, Viluksela M, Pohjanvirta R, Tuomisto J. The AH receptor and a novel gene determine acute toxic responses to TCDD: segregation of the resistant alleles to different rat lines. Toxicol Appl Pharmacol 1999;155:71–81.

[81] Pohjanvirta R, Wong JM, Li W, Harper PA, Tuomisto J, Okey AB. Point mutation in intron sequence causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-p-dioxin-resistant rat strain. Mol Pharmacol 1998;54:86–93.
[82] Poland A, Glover E, Kende AS. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for induction of aryl hydrocarbon hydroxylase. J Biol Chem 1976;251:4936–46.
[83] Pohjanvirta R, Viluksela M, Tuomisto JT, Unkila M, Karasinska J, Franc MA, et al. Physicochemical differences in the AH receptors of the most TCDD-susceptible and the most TCDD-resistant rat strains. Toxicol Appl Pharmacol 1999;155:82–95.

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Internal genomic sequence of human *CYP1A1* gene is involved in superinduction of dioxin-induced *CYP1A1* transcription by cycloheximide

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Received 26 January 2007
Available online 9 February 2007

Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces CYP1A1 mRNA expression, and co-treatment with the protein synthesis inhibitor, cycloheximide (CHX) magnifies its expression severalfold further. Although this phenomenon has been reported as superinduction, its molecular mechanism is still obscure. In the present study, we analyzed the influence of the *CYP1A1* internal genomic sequence on CHX-mediated superinduction. Partial sequences of the human *CYP1A1* were inserted at the 5′ end of the internal ribosomal entry site (IRES) connected to luciferase cDNA, and generated constructs were transiently transfected into CHO or HepG2 cells. Intron-1 deletion constructs showed higher inductivity than intron-1 intact constructs by TCDD. Quantitative RT-PCR analyses revealed that the superinduced levels by CHX of the intron-1 intact constructs were greater than those of the intron-1 deletion constructs. The present results indicate that internal genomic sequences of the human *CYP1A1* gene, especially the internal sequence of intron-1, are involved in superinduction of the *CYP1A1* gene by CHX.

Keywords: Superinduction; Cycloheximide; CYP1A1; Intron

The induction of cytochrome P450 (CYP) 1A1 causes metabolic activation of carcinogens and triggers DNA adducts [1,2]. CYP1A1 is also involved in wasting syndrome and hepatic dysfunction caused by high-dose dioxin exposure [3,4]. Because the response curve of *CYP1A1* gene induction determines the symptom levels of these various disorders, it is important to elucidate the mechanisms determining maximal and basal-level *CYP1A1* transcription.

It is well known that CYP1A1 induction is caused by ligand–AhR binding to the xenobiotic responsive element (XRE) located in the 5′-flanking transcriptional regulatory region [5]. In contrast to the extremely high-level CYP1A1 mRNA induction occurring in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposed cells, in normal cells *CYP1A1* transcription is nearly shut off. Therefore, it is believed that negative regulatory mechanisms exist. One candidate is a negative regulatory region located 5′ upstream of *CYP1A1* [6–8]. It has been reported that an Oct-1 motif existing −808 to −788 and a CT-rich tandem repeat sequence existing −818 to −791 were acting as negative regulatory elements (NRE) [9,10]. However, it is still unknown what is acting upon the element and whether or not the mechanism is involved in repressing the CYP1A1 basal level.

Unlike other genes, the induction of CYP1A1 can be further augmented by the protein synthesis inhibitor cycloheximide (CHX); this augmentation is known as superinduction [11,12]. This phenomenon suggests that the *CYP1A1* gene has a mechanism by which it avoids being induced at excessive levels. Monk and coworkers reported

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doi:10.1016/j.bbrc.2007.02.010

that *CYP1A1* gene transcription in rat primary keratinocytes is shut off by serial passage even at a high dose of TCDD exposure, and that CHX treatment recovered the transcription [13]. Because this silencing was not observed in stable transformant cells with 4.7 kb of the *CYP1A1* 5′-flanking region, they suggested that a labile repressor protein may affect elements located further upstream, downstream or within *CYP1A1* itself [14].

In the present study, to analyze the influence of *CYP1A1* internal sequences, we generated several constructs in which partial sequences of the human *CYP1A1* genome, each sequence containing 1.5 kb upstream of the 5′-flanking region, were inserted into the region upstream of the 5′ end of the internal ribosomal entry site (IRES) connected to luciferase cDNA. By employing a transient transfection assay system and direct measurement of the mRNA transcribed from the constructs, we found that *CYP1A1* intron-1 may contain some element involved in the superinduction by CHX.

Materials and methods

Materials. TCDD (purity > 99.5%) was purchased from Cambridge Isotope Laboratory (Andover, MA). 3-Methylcholanthrene (3MC, 98%) and cycloheximide (CHX) were from Aldrich Chemical (Milwaukee, WI). All reagents used for cell culture, RNA purification and reverse transcription, and Lipofectamine2000, were purchased from Invitrogen (Carlsbad, CA). The plasmid vectors (pCI-neo, pGL3-Basic, pRL-SV40) and Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). pIREs-hrGFP1a was purchased from Stratagene (La Jolla, CA). SuperFect Transfection Reagent was purchased from Qiagen (Hilden, Germany). SYBR Premix Ex Taq (Perfect Real Time) and TaKaRa LA Taq were purchased from Takara Bio Inc. (Otsu, Japan). Isogen was purchased from Nippon Gene Ltd. (Tokyo, Japan).

Cell culture. Human hepatoma cell lines (HepG2) and Chinese hamster ovarian cell lines (CHO) were grown in DMEM and Ham's F-12, supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.1% 2-mercaptoethanol. These cells were cultured in a humidified air incubator supplemented with 5% CO_2 at 37 °C. All assays were conducted by TCDD, 3MC, or TCDD plus CHX (added to medium 1 h prior to TCDD) exposure for 24 h.

Plasmid constructs. The CMV immediate-early enhancer/promoter of pCI-neo was removed and oligonucleotides containing the four restriction sites *Mlu*I, *Fse*I, *Eco*RI, and *Xho*I and a stop-codon cassette (sense: 5′-GA TCTACGCGTTTATTAGGCCGGCCGAATTCTAGGTGAGTAACTC GAGA-3′; anti-sense: 5′-CGCGTCTCGAGTTACTCACACCTAGAA TTCGGCCGGCCTAATAAACGCGT A-3′) were inserted. The *Xho*I–*Nco*I fragment containing IRES from pIRES-hrGFP-1a [15], the *Nco*I–*Xba*I fragment of *luciferase* from pGL3-Basic, were cut out and inserted into the modified pCI-neo, designated pIRESluc. The human *CYP1A1* gene was cloned by PCR amplification using LA Taq. The sequences were checked against GenBank Accession No.: AF253322 as described previously [16]. The region −1524 to +42 was amplified by PCR and inserted into pIRESluc at the *Mlu*I–*Eco*RI sites, and then pIRESluc-hcype1 (e1; Fig. 1) was generated. The regions −1524 to +2751 and −1524 to +4931 were removed between the *Mlu*I–*Fse*I and *Mlu*I–*Eco*RI sites and were inserted into pIRESluc to generate pIRESluc-hcype2 (e2) and pIRESluc-hcype7 (e7) (Fig. 1). By removing the 2029-bp sequence of intron-1 between the two *Xba*I sites, pIRESluc-hcype2x (e2x) and pIRESluc-hcype7x (e7x) were generated (Fig. 1). pCI-AhR, which expresses AhR, and ARNT expression vector pCI-ARNT were used to standardize the copy number of CYP1A1 from the constructs in real-time RT-PCR assays.

Transfection and luciferase reporter assay. Cells cultured in a 48-well multiplate to 100% confluence were used. HepG2 were cultured in antibiotic-free medium for 16 h and then transfected with 23.7 μM plasmid of pIRESluc-based constructs, 1 μg pCI-AhR, and 0.4 μg pRL-SV40 by adding 2 μl Lipofectamine2000 per well to the medium. CHO were transfected with 8.9 μM plasmid of pIRESluc-based constructs, 0.4 μg pCI-AhR, 0.2 μg pCI-ARNT, and 0.1 μg pRL-SV40 by adding 45 μl SuperFect Transfection Reagent per well to the medium. Chemical exposure to both cells was performed 24 h after the transfection. Reporter assays were conducted using the Dual-Luciferase Reporter Assay System as described previously [16].

Real-time and semiquantitative RT-PCR. The basic protocols used for real-time and semiquantitative RT-PCR have been reported [16]. All primer sets (shown in Table 1) were designed to differentiate specific and nonspecific products from genome DNA or plasmid. First, total RNA was extracted from cells using Isogen and treated with Amplification Grade DNase I. This RNA sample was reverse-transcribed using SuperScript III RNase H-Reverse Transcriptase and oligo(dT)12–18 primer. The cDNA sample was digested with restriction enzymes (Fig. 1; *Xba*I and *Pvu*II for CYP1A1-IRES-luciferase chimera mRNA; *Ban*I for CMV-AhR mRNA) to eliminate the amplification from contaminated plasmid DNAs and was then used for PCR analysis. Quantitative real-time RT-PCR was performed with SYBR Premix Ex Taq. All quantitative data were calculated by dividing the copy number of targets by the original RNA concentration. Semiquantitative RT-PCR analysis was conducted using LA Taq. The cycle conditions used are described in Table 1.

Results

Influence of CYP1A1 internal genomic sequences on transcription and superinduction from reporter plasmid in CHO cells

To examine intron-1's influence on the transcriptional activity and CHX-induced superinduction of *CYP1A1*, we generated five reporter constructs (Fig. 1) and employed the transient transfection system. First, we used a CHO in which endogenous hamster AhR was less active [17], with AhR and ARNT expression vectors. Transfected cells were exposed to serial concentrations of 3MC or 10 nM TCDD. By 3MC exposure, transcriptional activity from each of the constructs was induced in a dose-dependent manner (see Supplementary data). Although the length of *CYP1A1*-inserted constructs appeared to have a lesser degree of luciferase activity, e7x showed a higher induction level than e2 even though these constructs have nearly equal length. This trend was indistinguishable from the TCDD experiment (Fig. 2A). Next, RT-PCR was performed using Primer3 and 6 (Fig. 1). The products from e2 and e2x (407 bp), as well as those from e7 and e7x (1521 bp), showed the same band size by electrophoresis (Fig. 2B). Sequence analyses revealed that the CYP1A1-IRES-luciferase chimera primary transcript from these constructs, even though transiently transfected, were spliced at normal splice sites. Moreover, semiquantitative RT-PCR analyses using Primer1 and 4 showed that e2x and e7x induced greater levels of chimera mRNAs by TCDD exposure than did e2 and e7 (Fig. 2C). PCR using Primer8 and 9 was performed to amplify CMV-AhR chimera mRNA from pCI-AhR as internal standards. Next, real-time RT-PCR using these primer sets was conducted, and the results were directly proportional to those of the luciferase reporter assays

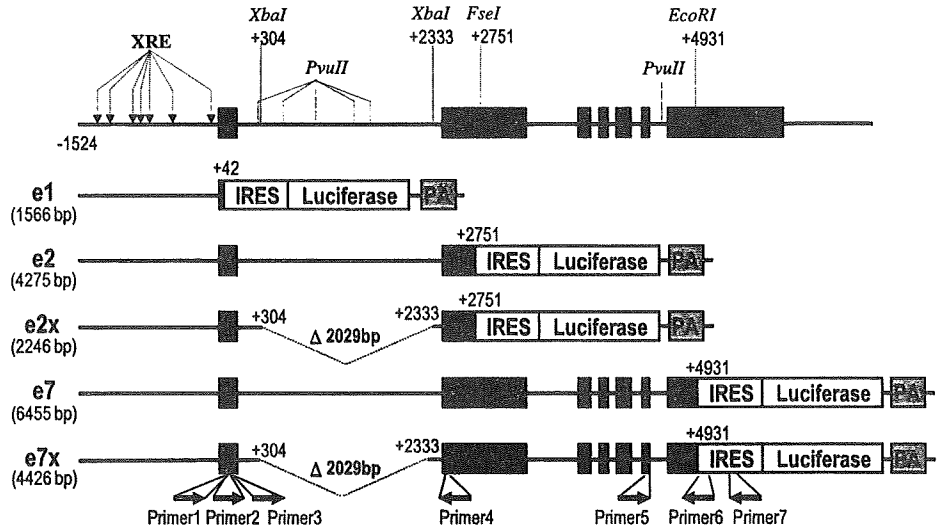

Fig. 1. Reporter constructs used in the present study. Five reporter constructs. The IRES-luciferase cassette was connected to the five partial *CYP1A1* genomic DNA, −1524 to +42 (e1), −1524 to +2751 (e2), −1524 to +4931 (e7), and the deletion of 2029-bp intron-1 (e2x, e7x). The restriction enzyme sites used are indicated above the upper line. The positions of Primer1–Primer7 used in the quantitative RT-PCR analysis are also indicated under the lower line.

Table 1
Primers used for semiquantitative or real-time quantitative RT-PCR

Targets	Primer sequences		Product size (bp)	Cycle used
	Forward (5′ to 3′)	Reverse (5′ to 3′)		
CYP1A1	CCAGGCTCCAAGAGTCCACC (Primer1)	GCCTTTGGGGACCTGAGG (Primer4)	183	32, LC
CYP-E1xIRES	AGGCTCCAAGAGTCCACCCTTC (Primer2)	GAGAGGGGGGTAACCCTATGCAG (Primer7)	1645 (531[a])	32, LC
	TTCCCAGCTCAGCTCAGTACCTCA (Primer3)	CCTTGTAGTCCTCGAGTTACTCAC (Primer6)	1521 (407[a])	
CYP-E6xIRES	AGGGGCGTTGTGTCTTTGT (Primer5)		366[b]	31, LC
CMV-AhR	GCTGCAGTGACTCTCTTAAGGTAGCC (Primer8)	CGCTGCTCATCTCGAGGCT (Primer9)	141	30, LC

LC: used for real-time RT-PCR with light cycler.
[a] In case of constructs e2 and e2x.
[b] Used with Primer7 as a reverse primer.

(Fig. 2D). These results proved that the reporter activities from constructs in CHO were in agreement with the quantities of CYP1A1 mRNA, and that intron-1 of human *CYP1A1* represses the transcriptional activity.

Next, transfected cells were exposed to 10 nM TCDD or 10 nM TCDD plus 30 μg/ml CHX. Real-time RT-PCR using the same primer sets (Fig. 3A) revealed that the superinduction levels of e2 (15.3-fold) and e7 (7.5-fold) were clearly higher than those of e2x (2.7-fold) and e7x (5.3-fold). These results were opposite those of the luciferase assay. Because the expression level of the CMV-AhR mRNA did not differ among the groups (Fig. 3B), these results suggested that the presence of intron-1 of human *CYP1A1* enhances superinduction by CHX.

Influence of CYP1A1 internal genomic sequences on transcription and superinduction from reporter plasmid in HepG2 cells

To examine whether or not this phenomenon in CHO experiments can be observed in human-derived cells, we next transfected these constructs into HepG2 transiently and exposed them to 10 nM TCDD for 24 h. The transcriptional activity increased as the length of the inserted *CYP1A1* region increased (Fig. 4A), which was the opposite of the result for CHO. However, e2x and e7x displayed higher luciferase activity than e2 and e7. Next, real-time RT-PCR using Primer2 and 7 for the e2 and e2x samples and Primer5 and 7 for the e7 and e7x samples was conducted, and the results were directly proportional to luciferase reporter assays (Fig. 4B). These results proved that the reporter activities from the constructs in HepG2 were in agreement with the quantities of CYP1A1 mRNA.

Next, transfected cells were exposed to 10 nM TCDD or 10 nM TCDD plus 30 μg/ml CHX. Real-time RT-PCR using the same primer sets (Fig. 4C) showed that the superinduction levels of e2 (13.9-fold) and e7 (13.4-fold) were clearly higher than those of e2x (4.5-fold) and e7x (3.9-fold), suggesting that the deletion of *CYP1A1* intron-1 decreased the level of superinduction. These results showed the same pattern as those of the CHO experiment, even though the results of the luciferase assay in CHO showed a different pattern than those in HepG2. The CMV-AhR mRNA expression level did not differ significantly among

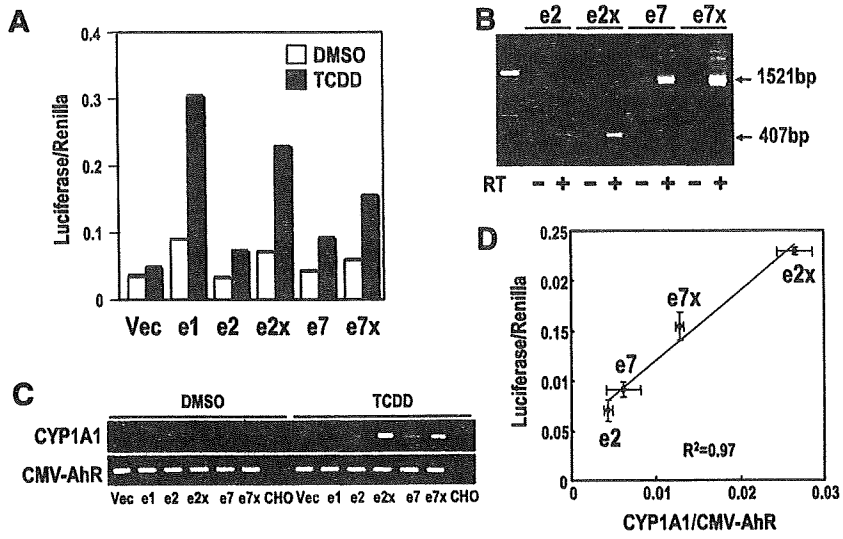

Fig. 2. Ligand-dependent transcriptional induction levels from five reporter constructs, transiently transfected to CHO. (A) TCDD (10 nM) induced transcriptional level induction. The data were expressed as firefly luciferase activity standardized by *Renilla* luciferase. (B) Confirmation of mRNA production from the reporter plasmid. (C) Comparison of CYP1A1 chimera mRNA expression levels from constructs by semiquantitative RT-PCR. (D) Relativity of reporter activity and mRNA level from each construct. The CYP1A1-IRES-luciferase chimera mRNA copy numbers standardized by CMV-AhR mRNA copy numbers from each construct were plotted with reporter activity obtained in (B). All data are expressed as means ± SD of triplicate assays.

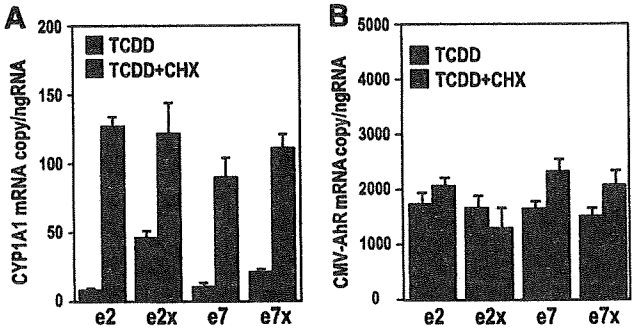

Fig. 3. Superinduction from each construct by CHX in CHO. (A) Comparison of CYP1A1 mRNA copy number from constructs. Using Primer1 and Primer2, CYP1A1-IRES-luciferase chimera mRNA copy numbers were calculated by real-time RT-PCR. (B) CMV-AhR mRNA copy numbers calculated by real-time RT-PCR analysis. All data are expressed as means ± SD of triplicate assays.

the transfection groups (Fig. 4D). These results suggested that the presence of intron-1 of human *CYP1A1* enhances the superinduction by CHX.

Discussion

In the present study, we generated pIRESluc vectors to analyze the influence of the *CYP1A1* internal genomic sequence on its own transcription. By means of a transient transfection assay in which we directly measured the expression levels of reporter plasmid mRNAs, we revealed that the *CYP1A1* internal genomic sequence is intensely involved in the superinduction mechanism activated by TCDD plus CHX. Notably, the deletion of intron-1 decreased superinduction levels in both HepG2 and CHO, suggesting that regulating factors for this phenomenon are preserved from primates to rodents.

One remarkable feature of our study was that we detected the superinduction of CYP1A1 mRNA by using reporter constructs even though they are transiently transfected into the cells. The present results ruled out the possibility that *CYP1A1* superinduction is involved in the change of chromatin texture [18].

The most accepted hypothesis about the mechanism of *CYP1A1* superinduction by CHX is that there should be an extremely short-half-life labile protein repressing the transcription of CYP1A1 [12,19]. Using murine hepatoma Hepa1c1c7 cells, the mechanism by which a labile protein represses the CYP1A1 transcriptional induction was suggested to involve the repression of liganded AhR degradation by CHX [20]. The authors of that study speculated that CHX prolongs the half-life of AhR and thereby promotes AhR activity. Because the proteasome inhibitor MG312 also repressed AhR degradation, they concluded that a labile repressor protein might promote the degradation of ubiquitinated AhR [21]. However, in a study using mammary gland epithelial carcinoma cells (MCF10A), *CYP1A1* superinduction was observed without ligand-specific AhR activation [22]. Thus, the existence of a labile repressor protein is still controversial.

In our preliminary experiments, we also detected superinduction of CYP1B1 and AhR repressor (AhRR) in HepG2 (see Supplementary data). Additionally, superinduction of TCDD inducible poly (ADP-ribose) polymerase (TiPARP) in mouse hepatoma Hepa1c1c7 has been reported [23]. All of these genes have XRE clusters upstream of

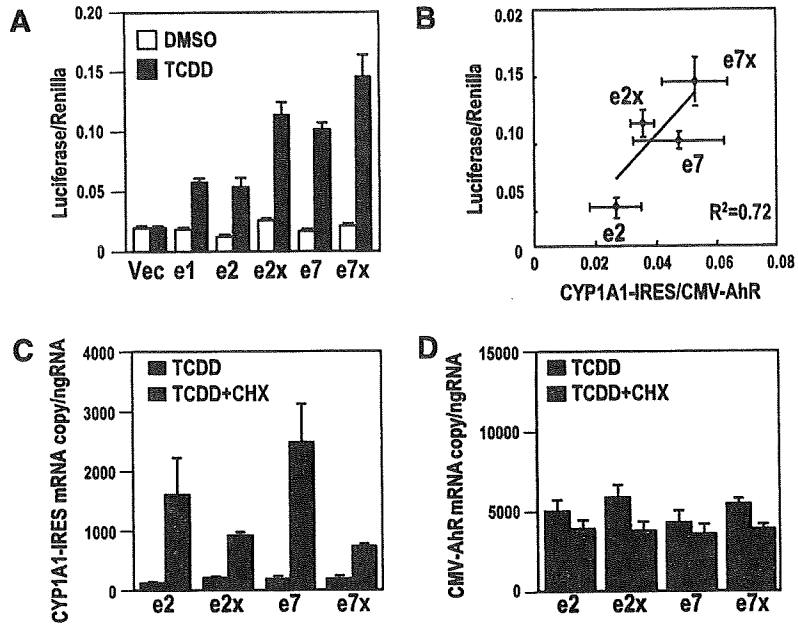

Fig. 4. Ligand-dependent transcriptional induction by TCDD and superinduction by TCDD plus CHX from five reporter constructs, transiently transfected to HepG2. (A) Comparison of 10 nM TCDD-induced transcriptional inductions. The data are expressed as firefly luciferase activity standardized by *Renilla* luciferase. (B) Relativity of reporter activity and mRNA level from each construct. The CYP1A1-IRES-luciferase chimera mRNA copy numbers standardized by CMV-AhR mRNA copy numbers from each construct were plotted with the reporter activity obtained in (A). (C) Comparison of CYP1A1 mRNA copy numbers from constructs. CYP1A1-IRES-luciferase chimera mRNA copy numbers were calculated by real-time RT-PCR. (D) CMV-AhR mRNA copy numbers calculated by real-time RT-PCR analysis. All data are expressed as means ± SD of triplicate assays.

the 5′-flanking region or in introns [24–26]. Therefore, it is plausible that superinduction requires only XRE, AhR, and the cofactors bound to AhR, including a labile repressor protein. However, this hypothesis cannot be applied to all AhR-induced genes. Recently, using HepG2 cells, it was proved that the XRE cluster near the 5′-upstream region of *CYP1A1*, 23 kb upstream from *CYP1A2*, has a bidirectional effect causing *CYP1A1* and *CYP1A2* induction by TCDD in HepG2 [27]. However, our results using HepG2 showed that TCDD plus CHX did not cause *CYP1A2* superinduction (see Supplementary data). Therefore, there should be a gene-specific mechanism of superinduction that can apply to CYP1A1 but not to CYP1A2.

Monk et al. suggested that a putative labile repressor protein affects the element that could be located further upstream, downstream, or within *CYP1A1* itself [13,14]. Therefore we generated pIRESluc constructs to investigate the interaction between internal genomic sequences and superinduction of *CYP1A1*. Our results strongly suggest that intron-1 has a repressive effect on CYP1A1 transcription after AhR activation, and that it is also involved in the superinduction by CHX.

The rat *CYP1A1* internal sequence in intron-1, GGAGGCACCTGGGAAAGCAGC, has been reported to have repression activity against transcription, as revealed by a CAT reporter with a 305-bp region of intron-1 connected to a Type II sodium channel minimum promoter [28]. This experiment was based on the fact that the sequence is very similar to the consensus sequence of the neuron-restrictive silencer factor (NRSF) binding site, NNCAGCACCNNGGACAGNNNC [29]. In addition, human CYP1A1 has a similar sequence at +1671, AGGAGGTACATGTGACAGCAGC, and DNase I hypersensitive mapping analysis showed the binding of some proteins to intron-1 [30]. Although there is no evidence as to whether NRSF protein is labile or not, this sequence may be involved in the superinduction.

In our results in CHO cells, the superinduction level of e7x (5.3-fold) was still higher than that of e2x (2.7-fold) (Fig. 3A). The present data suggest that internal genomic regions from exon-2 through exon-7 might be involved in the superinduction. Moreover, if the element of intron-1 is a unique NRE, superinduction should not be caused from e2x. But e2x showed a more than 2-fold increase in superinduction in CHO (Fig. 3A). Therefore, we hypothesized that a labile repressor protein is the factor acting not only on intron-1 but also on a multipoint element located in other introns and exons. Other genes that display superinduction may have consensus sequences, and exhaustive analysis of these genes would enable such sequences to identify the novel element we hypothesized. The vector we generated in this study would be useful for analyzing the interaction between internal genomic sequences and superinduction by CHX, and for discovering such a novel element.

Acknowledgments

The authors thank Dr. Kazuhiro Shiizaki for his technical assistance. This work was supported by the Environ-

mental Technology Development Fund (S.O.) from the Ministry of the Environment, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.010.

References

[1] A.H. Conney, Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture, Cancer Res. 42 (1982) 4875–4917.
[2] J.P. Whitlock Jr., Induction of cytochrome P4501A1, Annu. Rev. Pharmacol. Toxicol. 39 (1999) 103–125.
[3] D.W. Nebert, T.P. Dalton, A.B. Okey, F.J. Gonzalez, Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer, J. Biol. Chem. 279 (2004) 23847–23850.
[4] S. Uno, T.P. Dalton, P.R. Sinclair, N. Gorman, B. Wang, A.G. Smith, M.L. Miller, H.G. Shertzer, D.W. Nebert, Cyp1a1(−/−) male mice: protection against high-dose TCDD-induced lethality and wasting syndrome, and resistance to intrahepatocyte lipid accumulation and uroporphyria, Toxicol. Appl. Pharmacol. 196 (2004) 410–421.
[5] J. Mimura, Y. Fujii-Kuriyama, Functional role of AhR in the expression of toxic effects by TCDD, Biochim. Biophys. Acta 1619 (2003) 263–268.
[6] F.J. Gonzalez, D.W. Nebert, Autoregulation plus upstream positive and negative control regions associated with transcriptional activation of the mouse P1(450) gene, Nucleic. Acids Res. 13 (1985) 7269–7288.
[7] P.D. Boucher, R.J. Ruch, R.N. Hines, Specific nuclear protein binding to a negative regulatory element on the human CYP1A1 gene, J. Biol. Chem. 268 (1993) 17384–17391.
[8] K. Sterling, J. Weaver, K.L. Ho, L.C. Xu, E. Bresnick, Rat CYP1A1 negative regulatory element: biological activity and interaction with a protein from liver and hepatoma cells, Mol. Pharmacol. 44 (1993) 560–568.
[9] K. Sterling, E. Bresnick, Oct-1 transcription factor is a negative regulator of rat CYP1A1 expression via an octamer sequence in its negative regulatory element, Mol. Pharmacol. 49 (1996) 329–337.
[10] S.R. Nagy, M.S. Denison, Specificity of nuclear protein binding to a CYP1A1 negative regulatory element, Biochem. Biophys. Res. Commun. 296 (2002) 799–805.
[11] R.M. Teifeld, J.B. Fagan, D.S. Pasco, Transient superinducibility of cytochrome P450c (CYP1A1) mRNA and transcription, DNA 8 (1989) 329–338.
[12] A. Lusska, L. Wu, J.P. Whitlock Jr., Superinduction of CYP1A1 transcription by cycloheximide. Role of the DNA binding site for the liganded Ah receptor, J. Biol. Chem. 267 (1992) 15146–15151.
[13] S.A. Monk, M.S. Denison, R.H. Rice, Transient expression of CYP1A1 in rat epithelial cells cultured in suspension, Arch. Biochem. Biophys. 393 (2001) 154–162.
[14] S.A. Monk, M.S. Denison, R.H. Rice, Reversible stepwise negative regulation of CYP1A1 in cultured rat epidermal cells, Arch. Biochem. Biophys. 419 (2003) 158–169.
[15] S.K. Jang, E. Wimmer, Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein, Genes Dev. 4 (1990) 1560–1572.
[16] K. Shiizaki, S. Ohsako, T. Koyama, R. Nagata, J. Yonemoto, C. Tohyama, Lack of CYP1A1 expression is involved in unresponsiveness of the human hepatoma cell line SK-HEP-1 to dioxin, Toxicol. Lett. 160 (2005) 22–33.
[17] J. McGuire, K. Okamoto, M.L. Whitelaw, H. Tanaka, L. Poellinger, Definition of a dioxin receptor mutant that is a constitutive activator of transcription: delineation of overlapping repression and ligand binding functions within the PAS domain, J. Biol. Chem. 276 (2001) 41841–41849.
[18] O. Hankinson, Role of coactivators in transcriptional activation by the aryl hydrocarbon receptor, Arch. Biochem. Biophys. 433 (2005) 379–386.
[19] X. Ou, K.S. Ramos, Regulation of cytochrome P4501A1 gene expression in vascular smooth muscle cells through aryl hydrocarbon receptor-mediated signal transduction requires a protein synthesis inhibitor, Arch. Biochem. Biophys. 316 (1995) 116–122.
[20] Q. Ma, A.J. Renzelli, K.T. Baldwin, J.M. Antonini, Superinduction of CYP1A1 gene expression. Regulation of 2,3,7, 8-tetrachlorodibenzo-*p*-dioxin-induced degradation of Ah receptor by cycloheximide, J. Biol. Chem. 275 (2000) 12676–12683.
[21] Q. Ma, K.T. Baldwin, A cycloheximide-sensitive factor regulates TCDD-induced degradation of the aryl hydrocarbon receptor, Chemosphere 46 (2002) 1491–1500.
[22] A. Joiakim, P.A. Mathieu, A.A. Elliott, J.J. Reiners Jr., Superinduction of CYP1A1 in MCF10A cultures by cycloheximide, anisomycin, and puromycin: a process independent of effects on protein translation and unrelated to suppression of aryl hydrocarbon receptor proteolysis by the proteasome, Mol. Pharmacol. 66 (2004) 936–947.
[23] Q. Ma, Induction and superinduction of 2,3,7,8-tetrachlorodibenzo-rho-dioxin-inducible poly(ADP-ribose) polymerase: role of the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator transcription activation domains and a labile transcription repressor, Arch. Biochem. Biophys. 404 (2002) 309–316.
[24] O. Dohr, R. Sinning, C. Vogel, P. Munzel, J. Abel, Effect of transforming growth factor-beta1 on expression of aryl hydrocarbon receptor and genes of Ah gene battery: clues for independent down-regulation in A549 cells, Mol. Pharmacol. 51 (1997) 703–710.
[25] J. Mimura, M. Ema, K. Sogawa, Y. Fujii-Kuriyama, Identification of a novel mechanism of regulation of Ah (dioxin) receptor function, Genes Dev. 13 (1999) 20–25.
[26] Q. Ma, K.T. Baldwin, A.J. Renzelli, A. McDaniel, L. Dong, TCDD-inducible poly(ADP-ribose) polymerase: a novel response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, Biochem. Biophys. Res. Commun. 289 (2001) 499–506.
[27] R. Ueda, H. Iketaki, K. Nagata, S. Kimura, F.J. Gonzalez, K. Kusano, T. Yoshimura, Y. Yamazoe, A common regulatory region functions bidirectionally in transcriptional activation of the human CYP1A1 and CYP1A2 genes, Mol. Pharmacol. 69 (2006) 1924–1930.
[28] R. Garcia-Sanchez, J. Ayala-Lujan, A. Hernandez-Perez, T. Mendoza-Figueroa, J. Tapia-Ramirez, Identification of repressor element 1 in cytochrome P450 genes and their negative regulation by RE1 silencing transcription factor/neuron-restrictive silencer factor, Biochim. Biophys. Acta 1620 (2003) 39–46.
[29] C.J. Schoenherr, A.J. Paquette, D.J. Anderson, Identification of potential target genes for the neuron-restrictive silencer factor, Proc. Natl. Acad. Sci. USA 93 (1996) 9881–9886.
[30] K. Gradin, A. Wilhelmsson, L. Poellinger, A. Berghard, Nonresponsiveness of normal human fibroblasts to dioxin correlates with the presence of a constitutive xenobiotic response element-binding factor, J. Biol. Chem. 268 (1993) 4061–4068.

Doi: 10.1111/j.1742-7843.2008.00309.x
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Omeprazole Alleviates Benzo[a]pyrene Cytotoxicity by Inhibition of CYP1A1 Activity in Human and Mouse Hepatoma Cells

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(Received March 27, 2008; Accepted June 2, 2008)

Abstract: Omeprazole is a drug used for treating gastro-oesophageal reflux disease and duodenal ulcers. Omeprazole induces a xenobiotic-metabolizing enzyme, cytochrome P450 1A1 (CYP1A1), as its ligand by aryl hydrocarbon receptor (AhR) activation without binding. CYP1A1-inducible chemicals, such as benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, are known to have adverse effects (i.e. carcinogenesis, mutagenesis and malformation). Unlike these typical AhR activators, omeprazole has shown no experimental evidence of carcinogenic activity. The possibility, however, remains that omeprazole may aggravate the effect of environmental carcinogens through CYP1A1 induction. We exposed benzo[a]pyrene and omeprazole simultaneously to human and mouse hepatoma cells to investigate the synergistic effect of these chemicals. Contrary to our prediction, cytotoxicity of benzo[a]pyrene was inhibited by the omeprazole exposure in a dose-dependent manner. Omeprazole did not alter CYP1A1 mRNA and protein levels induced by benzo[a]pyrene. The 7-ethoxy-resorufin-O-deethylase assay revealed that omeprazole inhibited CYP1A1 enzyme activity. Kinetic analysis also demonstrated that it is a competitive inhibitor for CYP1A1. The K_m value of omeprazole against CYP1A1 activity was 50.1 μM. We conclude that the effects of omeprazole on CYP1A1 involve not only induction through AhR activation but also inhibition of its enzyme activity, and that the protective effect of omeprazole against benzo[a]pyrene cytotoxicity depends on the latter.

Omeprazole, a benzimidazole derivative, is a potent suppressor of gastric acid secretion [1] and has been used for treating gastro-oesophageal reflux disease and duodenal ulcers. Omeprazole is known to have two distinct effects on drug metabolic enzymes: as an inhibitor of cytochrome P450 2C19 (CYP2C19) and as an inducer of CYP1A1 [2,3], and it also shows adverse abilities of both induction and inhibition against CYP3A4 [4].

CYP1A1 is known as a xenobiotic-metabolizing enzyme, and its transcriptional activation is mediated by aryl hydrocarbon receptor (AhR) [5,6]. AhR is identified as a ligand-activated transcription factor and member of the basic helix–loop–helix super family protein. Following the binding to ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, AhR translocates to the nucleus and forms a heterodimer with AhR nuclear translocator [7]. The AhR/AhR nuclear translocator complex enhances the transcription of genes for phase I drug-metabolizing enzymes, including CYP1A1, 1A2 and 1B1 [8]. As AhR ligands such as benzo[a]pyrene are metabolized by CYP1A1, one of the functions of AhR is presumed to be that of a sensor for environmental chemicals or drugs [9].

Interestingly, unlike the typical AhR activator, omeprazole induces CYP1A1 without binding to AhR as a ligand [10]. Because CYP1A1 induction by omeprazole requires AhR, omeprazole has been thought to activate AhR through a different pathway from ligand binding [11]. The omeprazole-mediated AhR activation is reported to be species-specific. CYP1A1 induction by omeprazole has been shown in primary hepatocytes or hepatoma cell lines of man but not of mouse [12]. The omeprazole-mediated CYP1A1 induction requires more than 12 hr after the omeprazole treatment, which is slower compared to the benzo[a]pyrene-mediated induction [13]. Some reports suggest that protein phosphorylation is correlated to the omeprazole-mediated AhR activation, because some protein kinase inhibitors can block the activation [14,15]. However, the molecular mechanisms of omeprazole-mediated AhR activation are still unclear.

Some CYP1A1-inducible chemicals are known to produce adverse effects, such as carcinogenesis, mutagenesis and malformation. Among these chemicals, benzo[a]pyrene binds to the aryl hydrocarbon receptor as a ligand and induces CYP1A1 expression [16]. Subsequently, it is metabolized by CYP1A1 to electrophilic species, such as benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide, which can eventually produce bulky DNA adducts or oxidative DNA damage [17]. In addition, benzo[a]pyrene and other polyaromatic hydrocarbons have been shown to induce apoptosis in Hepa-1c1c7 cells [18]. CYP1A1 induction plays a key role in these toxic events, because CYP1A1- or AhR-deficient sub-lines derived from Hepa-1c1c7 cells show resistance against benzo[a]pyrene exposure [19].

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