hydrocarbon receptor-dependent nuclear translocation of NF-κB and expression of Fas ligand in thymic stromal cells and consequent apoptosis in T cells. *J. Immunol.* 175: 90–103.
33. White, S. C., and G. W. Casarett. 1974. Induction of experimental autoallergic sialadenitis. *J. Immunol.* 112: 178–185.
34. Kohashi, M., N. Ishimaru, R. Arakaki, and Y. Hayashi. 2008. Effective treatment with oral administration of rebamipide in a mouse model for Sjogren's syndrome. *Arthritis Rheum.* 58: 389–400.
35. Rengarajan, J., S. J. Szabo, and L. H. Glimcher. 2000. Transcriptional regulation of Th1/Th2 polarization. *Immunol. Today* 21: 479–783.
36. Riddick, D. S., Y. Huang, P. A. Harper, and A. B. Okey. 1994. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin versus 3-methylcholanthrene: comparative studies of Ah receptor binding, transformation, and induction of CYP1A1. *J. Biol. Chem.* 269: 12118–12128.
37. Singh, N. P., M. Nagarkatti, and P. S. Nagarkatti. 2007. Role of dioxin response element and nuclear factor-κB motifs in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated regulation of Fas and Fas ligand expression. *Mol. Pharmacol.* 71: 145–157.
38. Bonizzi, G., and M. Karin. 2004. The two NF-κB activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* 25: 280–288.
39. Anderson, M. S., E. S. Venanzi, L. Klein, Z. Chen, S. P. Berzins, S. J. Turley, H. von Boehmer, R. Bronson, A. Dierich, C. Benoist, and D. Mathis. 2002. Protection of an immunological self shadow within the thymus by the aire protein. *Science* 298: 1395–1401.
40. Kuroda, N., T. Mitani, N. Takeda, N. Ishimaru, R. Arakaki, Y. Hayashi, Y. Bando, K. Izumi, T. Takahashi, T. Nomura, et al. 2005. Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of Aire-deficient mice. *J. Immunol.* 174: 1862–1870.
41. Ito, T., S. Tsukumo, N. Suzuki, H. Motohashi, M. Yamamoto, Y. Fujii-Kuriyama, J. Mimura, T. M. Lin, R. E. Peterson, C. Tohyama, and K. Nohara. 2004. A constitutively active arylhydrocarbon receptor induces growth inhibition of Jurkat T cells through changes in the expression of genes related to apoptosis and cell cycle arrest. *J. Biol. Chem.* 279: 25204–25210.
42. Nagarkatti, P. S., G. D. Sweeney, J. Gauldie, and D. A. Clark. 1984. Sensitivity to suppression of cytotoxic T cell generation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is dependent on the Ah genotype of the murine host. *Toxicol. Appl. Pharmacol.* 72: 169–176.
43. Nohara, K., X. Pan, S. Tsukumo, A. Hida, T. Ito, H. Nagai, K. Inouye, H. Motohashi, M. Yamamoto, Y. Fujii-Kuriyama, and C. Tohyama. 2005. Constitutively active aryl hydrocarbon receptor expressed specifically in T-lineage cells causes thymus involution and suppresses the immunization-induced increase in splenocytes. *J. Immunol.* 174: 2770–2777.
44. Staples, J. E., F. G. Murante, N. C. Fiore, T. A. Gasiewicz, and A. E. Silverstone. 1998. Thymic alterations induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are strictly dependent on aryl hydrocarbon receptor activation in hemopoietic cells. *J. Immunol.* 160: 3844–3854.
45. Ishimaru, N., T. Yoneda, K. Saegusa, K. Yanagi, N. Hanejі, K. Moriyama, I. Saito, and Y. Hayashi. 2000. Severe destructive autoimmune lesions with aging in murine Sjögren's syndrome through Fas-mediated apoptosis. *Am. J. Pathol.* 156: 1557–1564.
46. Kobayashi, M., N. Yasui, N. Ishimaru, R. Arakaki, and Y. Hayashi. 2004. Development of autoimmune arthritis with aging via bystander T cell activation in the mouse model of Sjögren's syndrome. *Arthritis Rheum.* 50: 3974–3984.
47. Kerkvliet, N. I., and J. A. Brauner. 1987. Mechanisms of 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (HpCDD)-induced humoral immune suppression: evidence of primary defect in T-cell regulation. *Toxicol. Appl. Pharmacol.* 87: 47–58.
48. Luster, M. I., D. R. Germolec, G. Clark, G. Wiegand, and G. J. Rosenthal. 1998. Selective effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and corticosteroid on in vitro lymphocyte maturation. *J. Immunol.* 140: 928–935.
49. Schmidt, J. V., G. H. Su, J. K. Reddy, M. C. Simon, and C. A. Bradfield. 1996. Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development. *Proc. Natl. Acad. Sci. USA* 93: 6731–6736.
50. Gu, Y. Z., J. B. Hogenesch, and C. A. Bradfield. 2000. The PAS superfamily: sensors of environmental and developmental signals. *Annu. Rev. Pharmacol. Toxicol.* 40: 519–561.
51. Umannova, L., J. Zatloukalova, M. Machala, P. Kremar, Z. Majkova, B. Hennig, A. Kozubik, and J. Vondracek. 2007. Tumor necrosis factor-α modulates effects of aryl hydrocarbon receptor ligands on cell proliferation and expression of cytochrome P450 enzymes in rat liver "stem-like" cells. *Toxicol. Sci.* 99: 79–89.
52. Hoagland, M. S., E. Hoagland, and H. I. Swanson. 2005. The p53 inhibitor pifithrin-α is a potent agonist of aryl hydrocarbon receptor. *J. Pharmacol. Exp. Ther.* 314: 603–610.
53. Vogel, C. F., E. Sciullo, W. Li, P. Wong, G. Lazennec, and F. Matsumura. 2007. RelB, a new partner of aryl hydrocarbon receptor-mediated transcription. *Mol. Endocrinol.* 21: 2941–2955.
54. Vogel, C. F., E. Sciullo, and F. Matsumura. 2007. Involvement of RelB in aryl hydrocarbon receptor-mediated induction of chemokines. *Biochem. Biophys. Res. Commun.* 363: 722–726.
55. Gillard, G. O., and A. G. Farr. 2005. Contrasting models of promiscuous gene expression by thymic epithelium. *J. Exp. Med.* 202: 15–19.
56. Mandal, P. K. 2005. Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology. *J. Comp. Physiol.* 175: 221–230.
57. Fan, F., B. Yan, G. Wood, M. Vilukscla, and K. K. Rozman. 1997. Cytokines (IL-1β and TNFα) in relation to biochemical and immunological effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rats. *Toxicology* 116: 9–16.
58. Vogel, C., S. Donat, O. Döhr, L. Kremer, C. Esser, M. Roller, and J. Abel. 1997. Effect of subchronic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure on immune system and target gene responses in mice: calculation of benchmark doses for CYP1A1 and CYP1A2 related enzyme activities. *Arch. Toxicol.* 71: 372–382.
59. Warren, T. K., K. A. Mitchell, and B. P. Lawrence. 2000. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) suppresses the humoral and cell-mediated immune responses to influenza A virus without affecting cytolytic activity in the lung. *Toxicol. Sci.* 56: 114–123.
60. Veldhoen, M., K. Hirota, A. M. Westendorf, J. Buer, L. Dumoutier, J. C. Renauld, and B. Stockinger. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453: 106–110.
61. Quintana, F. J., A. S. Basso, A. H. Iglesias, T. Korn, M. F. Farez, E. Bettelli, M. Caccamo, M. Oukka, and H. L. Weiner. 2008. Control of Treg and TH17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453: 65–72.

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.34, Special Issue II, SP199-SP200, 2009

Overview: "Children's Toxicology", a renovating study field of irreversible "early exposure-delayed effects"

Jun Kanno

Division of Cellular and Molecular Toxicology, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

(Received February 17, 2009)

ABSTRACT — "Children are not small adults". This is a well-known phrase, especially in the clinics for diagnosis, efficacy of treatment, side effect, and prognosis. However, in the field of toxicology, this issue has long been a challenge. The knowledge has been limited to the differences in metabolism and other physiological factors. Currently available test guidelines for fetuses and immature animals are teratogenicity and reproductive toxicity studies. These tests look for straight-forward (essentially macroscopic) outcomes established within a rather short period of exposure to the test substances. However, recent advances in molecular toxicology allow combination of *in vitro* and *in vivo* studies at molecular levels. The target molecules and receptors can be identified in quantitative fashion and at the fine structure levels around and below the resolution of normal light microscopy. Such expansion of the knowledge lead us to consider a rather new category of "receptor mediated toxicity" or "signal toxicity". Such non-organic insults would merely induce transient effects on adults. However, there are growing evidences that such slight insults on the developing and maturating organisms can leave irreversible effects that become overt in adulthood. As an overview, toxicology has entered a new phase where children's toxicology becomes a renovating study field of the irreversible "early exposure-delayed effects".

Key words: Children's toxicology, Receptor-mediated toxicity, Signal toxicity, Early exposure-delayed effect

INTRODUCTION

Toxicology is a study to analyze interaction between living organisms and xenobiotics, and its final goal is to secure the safety of humans and environment in modern life where various products and technologies are used. Up to now, the majority of toxicological tests to evaluate the toxicity of a particular substance are utilizing experimental animals as a surrogate of humans. The results obtained from such animal tests are extrapolated to humans for the settlement of various kinds of regulation on the test substances, i.e. food additives, pesticides, industrial chemicals, medicines, etc. In cases of pharmaceutical products, clinical trials (human tests) are available. However, these are rather exceptional occasions for toxicology as a whole. It would be very difficult for non-pharmaceutical objects to test on humans, and even for pharmaceuticals, human trial for children including fetuses have many difficulties.

Current toxicological testing protocols are based on an assumption that both experimental animals and humans share common basic structure of the body and thus similar biological reaction. Most of those toxicological studies are based on "diagnosis" of the symptoms of experimental animals in a similar fashion to give a diagnosis to human patients. Because the fine structure and function of the bodies are still unknown, both humans and animal bodies are "black boxes" responding to the test substances by showing various symptoms. Usually, the "no observed adverse effect level" (NOAEL) or "no observed effect level" (NOEL) is given by such tests. Since the basic nature of species differences and individual differences are not known, a number called "safety factor" was invented to extrapolate animal NOAEL/NOEL data to humans (Benford, 2000). Normally, a factor of 10 for the species and another 10 for individual differences, thus 100 as a whole, is used to set lower NOAEL/NOEL levels for humans. This approach has been working well for the majority of test substances. Not surprisingly, however, there are some exceptions. Thalidomide is a best-known example (Newman, 1985). Phocomelia, a spectrum of malformation of limbs, was induced in offspring of tha-

Correspondence: Jun Kanno (E-mail: kanno@nihs.go.jp)

lidomide-treated pregnant women, but not observed in offspring of mice and rats. Therefore, more precise toxicity evaluation/prediction is obviously needed for safer assessment. An approach that enables us to point out molecular mechanisms of toxicity would be essential for such needs and to better understand the species-specific responses.

DISCUSSION

To modernize the toxicology and improve the accuracy of safety assessments, we are attempting to describe and understand the organism-xenobiotics interaction at the molecular levels. Different for other exploratory studies, a major prerequisite is that the Toxicology must be prepared for any unexpected or unpredictable responses. Thus, the approach must be comprehensive. Consequently, we adopted a whole-genome cDNA microarray system for a comprehensive monitoring of the transcriptome, and launched the Percellome Toxicogenomics Project, of which the ultimate goal is to illustrate out the whole regulatory pathways induced by xenobiotics in the experimental animals, mainly mice, including embryo (Kanno *et al.*, 2006).

On top of that, there is an important factor of toxicology, that it the "time frame" such as acute, chronic and delayed toxicity. Among them, researches for the assessment of delayed toxicity targeted for children (including fetus and infants) is becoming very important. It is very likely that the children have a chance to be exposed in daily life to a series of substances which can be a cause of delayed toxicity, especially, of the highly evolved systems, that is endocrine, immune and central nervous system. Such chemical substances can affect the developing systems at a dosage lower than the dosage that induces overt cytocidal changes that would link to immediate appearance of symptoms. For example, our recent experience on the perinatal exposure study (Tanemura *et al.*, 2009) which resulted in the emergence of delayed effects on neurobehavioral endpoints can be explained by a metaphor. That is, "No one turns on power when building a computer, but the living brains are built under the "power-on" situation". It is very likely that the developing brain needs proper or normal signals to build up its fine structures and functional networks (Cohen-Cory, 2002). At this stage, if the signals are disrupted by exogenous insults, it may result in malformation of the fine structure of the brain system. In this case, it is not necessary to directly kill the nerve cells during exposure. The malformation of fine structure/functional network will become symptomatic when the animals grow up to adults. On the other hand, most of those insults to adults would end up in reversible and transient changes.

Such delayed toxicity cannot be readily detected by currently available functional observational battery-(FOB-) based neuronal test system. Our new findings fall into the category of "early exposure- delayed effect". As mentioned above, nervous systems of developing organisms are susceptible to signal disruption which could lead to the delayed neurobehavioral anomaly. Toxicology is asked to prepare to respond to such new types of toxicity or "signal toxicity" with a consideration on the mechanisms which could explain the severity and irreversibility specific to children.

In conclusion, the 35th Annual Meeting of the Japanese Society of Toxicology had raised "Children's Toxicology" as one of its main Themes, and organized Special lectures, five Symposiums and two Workshops on Children's Toxicology of various targets and pending problems, which includes central nervous system, immune system, and endocrine system as targets, as well as problems in pharmacology i.e. issues on children's preclinical and clinical trials and on the off-label use of drugs. This special issue of the Journal of Toxicological Sciences gathers the peer-reviewed papers presented by the authors who participated in the lectures/symposiums/workshops on Children's Toxicology at the meeting.

REFERENCES

Benford, D. (2000): The Acceptable Daily Intake: A food for ensuring food safety. ILSI Europe Concise Monograph series. Brussels, Belgium.

Cohen-Cory, S. (2002): The developing synapse: construction and modulation of synaptic structures and circuits. Science, **298**, 770-776.

Kanno, J., Aisaki, K., Igarashi, K., Nakatsu, N., Ono, A., Kodama, Y. and Nagao, T. (2006): "Per cell" normalization method for mRNA measurement by quantitative PCR and microarrays. BMC Genomics, 7, 64.

Newman, C.G.H. (1985): Teratogen update: clinical aspects of thalidomide embryopathy-a continuing preoccupation. Teratology, **32**, 133-44.

Tanemura, K., Igarashi, K., Matsugami, T.R., Aisaki, K., Kitajima, S. and Kanno, J. (2009): Brain structure impairment and behavioral disturbance induced in male mice offspring by a single intraperitoneal administration of domoic acid (DA) to their dams. J. Toxicol. Sci., **34**, Special Issue II, SP279-SP286.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 17, pp. 11017–11026, April 24, 2009

SUMO Modification Regulates the Transcriptional Repressor Function of Aryl Hydrocarbon Receptor Repressor*[S]

Received for publication, November 17, 2008, and in revised form, February 27, 2009 Published, JBC Papers in Press, February 27, 2009, DOI 10.1074/jbc.M808694200

Motohiko Oshima[‡§], Junsei Mimura[¶], Hiroki Sekine[‡§], Hiromi Okawa[‡§], and Yoshiaki Fujii-Kuriyama[‡§1]

From the [‡]Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan, the [¶]Department of Stress Response Science, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori 036-8562, Japan, and [§]Solution Oriented Research for Science and Technology, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi 332-0012, Japan

The aryl hydrocarbon receptor (AhR) repressor (AhRR) inhibits the AhR activity. AhRR acts by competing with AhR for heterodimer formation with the AhR nuclear translocator (Arnt) and preventing the AhR·Arnt complex from binding the xenobiotic-responsive elements. Here, we report that AhRR has three evolutionarily conserved SUMOylation consensus sequences within its C-terminal repression domain and that Lys-542, Lys-583, and Lys-660 at the SUMOylation sites are modified by SUMO-1 *in vivo*. Arginine mutation of the three lysines results in a significant reduction of transcriptional repression activity. SUMOylation of the three lysine residues is important for the interaction between AhRR and ANKRA2, HDAC4, and HDAC5, which are important corepressors for AhRR. Arnt, a heterodimer partner for AhRR, markedly enhanced the SUMOylation of AhRR. AhRR, but not AhR, also significantly enhanced the SUMOylation of Arnt. The SUMOylation of both AhRR and Arnt is important for the efficient transcriptional repression activity of the AhRR/Arnt heterodimer.

The aryl hydrocarbon receptor repressor (AhRR)[2] is a member of the bHLH-PAS (basic helix-loop-helix and the Per-Arnt-Sim) protein superfamily and has a high similarity to AhR in the N-terminal bHLH-PAS A domain (1). The 5′-flanking promoter region of the mouse AhRR gene contains conserved xenobiotic-responsive element (XRE) sequences, and expression of the AhRR gene is induced by binding of the AhR/Arnt heterodimer to these XREs (2). AhRR forms a heterodimer with Arnt, another bHLH-PAS transcription factor, to inhibit AhR-dependent transactivation of the XRE-driven genes; thus, AhRR participates in a negative feedback loop in the AhR signaling pathway (1, 3, 4). Recently, we generated AhRR$^{-/-}$ mice, which show higher than wild type levels of ligand-induced expression of the AhR target gene, *Cyp1a1* mRNA induction in some tissues (5). These mice also displayed a delayed response to skin carcinogenesis caused by benzo[a]pyrene (5). Recent work has also demonstrated that AhRR is a tumor suppressor gene (6); AhRR mRNA is consistently down-regulated in human malignant tissues from different anatomical origins; furthermore, ectopic expression of AhRR in tumor cells resulted in diminished cell growth (7) and reduced angiogenic potential. These observations provide new insight into the still largely unknown physiological functions of AhRR and form the basis for further studies of its mechanisms of action.

We reported previously that AhRR contains a transcriptional repression domain within its C-terminal region (8). Using the C-terminal region of AhRR as bait, we isolated ANKRA2 (ankyrin repeat, family A, 2) by CytoTrap yeast two-hybrid screening. AhRR was also shown to interact with HDAC4 and HDAC5, and these interactions are important for the transcriptional repression activity of AhRR (8).

Small ubiquitin-like modifiers (SUMO-1, SUMO-2, and SUMO-3) belong to a family of ubiquitin-like proteins, which are covalently attached to (or detached from) substrate proteins to regulate their functions. The post-translational modification of proteins by SUMO has been increasingly recognized as an important regulatory mechanism in a diverse range of cellular processes. SUMO precursors are processed by SUMO-specific proteases and activated by an E1 enzyme. The activated SUMO is transferred to the E2 conjugating enzyme Ubc9, which recognizes the SUMOylation consensus sequence ψKXE within target proteins. With the help of E3 ligases such as PIAS, the C-terminal glycine of SUMO is covalently linked to the ε-amino group of lysine in the SUMOylation consensus sequence of the target proteins (9, 10).

In contrast to ubiquitylation, SUMOylation has a wide range of substrate-specific functions and acts via multiple mechanisms, including alterations in the subcellular localization of target proteins, protein stability, protein-protein interactions, and protein-DNA binding activities. Many transcription factors are SUMOylated. In many cases, SUMO modification of transcription factors is associated with transcriptional repression through the suppression of transactivation activity or the enhancement of repression activity.

In this study, we report that three lysine residues within the C-terminal repression domain of AhRR can be modified by

* This work was supported in part by a scientific research grant from the Ministry of Health, Labor, and Welfare of Japan and Solution Oriented Research for Science and Technology, Japan Science and Technology Agency.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

[1] To whom correspondence should be addressed: Center for Tsukuba Advanced Research Alliance, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8577, Japan. Tel.: 81-29-853-7323; Fax: 81-29-853-7318; E-mail: ykfujii@tara.tsukuba.ac.jp.

[2] The abbreviations used are: AhRR, aryl hydrocarbon receptor repressor; AhR, aryl hydrocarbon receptor; SUMO, small ubiquitin-like modifier; WT, wild type; EGFP, enhanced green fluorescent protein; XRE, xenobiotic-responsive element; HA, hemagglutinin; m, mouse; Arnt, AhR nuclear translocator; GAL4DBD, GAL4 DNA-binding domain; 3-MC, 3-methyl cholanthrene; PML, promyelocytic leukemia.

SUMO-1, resulting in enhancement of its transcriptional repression activity.

EXPERIMENTAL PROCEDURES

Plasmids—Constructions of pBOSGAL4DBD-AhRR, pBOSHA-AhRR, pBOSFLAG-ANKRA2, pG3TK-Luc, and plasmids encoding HDAC4-FLAG and HDAC5-FLAG was described previously (8). pSG5His-SUMO-1 was generated by fusing full-length human *SUMO-1* cDNA with the His tag sequence and cloning into the expression vector pSG5 (Stratagene). pBOSHA-SUMO-1 and pBOSHA-SUMO-2 were generated by ligating the full-length human *SUMO-1* or *SUMO-2* cDNA with the SmaI site of pBOST7-HA. pBOS-Ubc9 was generated by inserting the EcoRI/SalI fragment of pBSK-mUbc9 into the pEFBOS vector (11) cleaved with EcoRI/SalI. pCMV3xFLAG-AhRR was constructed by ligating the blunt-ended EcoRI/SalI fragment from pBSK-mAhRR with the blunt-ended p3xFLAG-CMV-10 (Sigma). AhRR was cleaved from pBSK-AhRR (8) with EcoRI/XhoI, blunt-ended, and subsequently inserted into the EGFP-C3 vector, which was cut with SmaI to generate pEGFP-AhRR. pBOS-AhR, pBOS-Arnt, and pXRE4TK-Luc were described previously (1). To construct pBOSHA-AhR and pBOSHA-Arnt, pBSK-mAhR and pBSK-mArnt (1) were digested with HindIII/XbaI and NcoI/BamHI, respectively, and the blunt-ended fragments of mAhR and mArnt were ligated with the SmaI site of pBOST7-HA. pBOS3xFLAG-Arnt was described previously (12). Single, double, or triple amino acid mutations in AhRR (K542R, K583R, K660R) or Arnt (K245R) were generated using the QuikChange site-directed mutagenesis kit (Stratagene).

Cell Culture and Transfection—COS-7, Hepa-1c1c7 (Hepa-1), and HeLa cells were maintained, respectively, in high or low glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (Invitrogen) under 5.0% CO_2 at 37 °C. Transfection was performed using Lipofectamine™ (Invitrogen).

Luciferase Assay—Hepa-1, COS-7, or HeLa cells (5.0×10^4 cells/well) were grown in 24-well dishes for 24 h and transfected with the expression plasmids indicated in the figure legends, such as pG3TK-Luc or pXRE4TK-Luc, and the expression plasmids for sea pansy luciferase as an internal control. Cells transfected with pXRE4TK-Luc were treated with 2 μM 3-MC or Me_2SO for 12 h. Forty eight h after transfection, the cells were harvested, and luciferase was quantified using the Dual-Luciferase reporter assay system (Promega). Expressed firefly luciferase activity was normalized to the cotransfected sea pansy luciferase activity, which was used as a standard.

Co-immunoprecipitation and Immunoblot Analysis—The transfected COS-7 cells were lysed in radioimmune precipitation assay buffer for immunoprecipitation with the anti-HA antibody or in FLAG buffer for immunoprecipitation with the anti-FLAG antibody; buffer contained protease inhibitor mixture (Roche Applied Science) and 20 mM *N*-ethylmaleimide to preserve the SUMOylation. Whole cell lysates were used for immunoblot analysis either directly or after immunoprecipitation. Immunoprecipitation with anti-FLAG M2 agarose (Sigma) or anti-HA agarose (Sigma) was performed for 12 h, and the immunoprecipitates were washed according to the

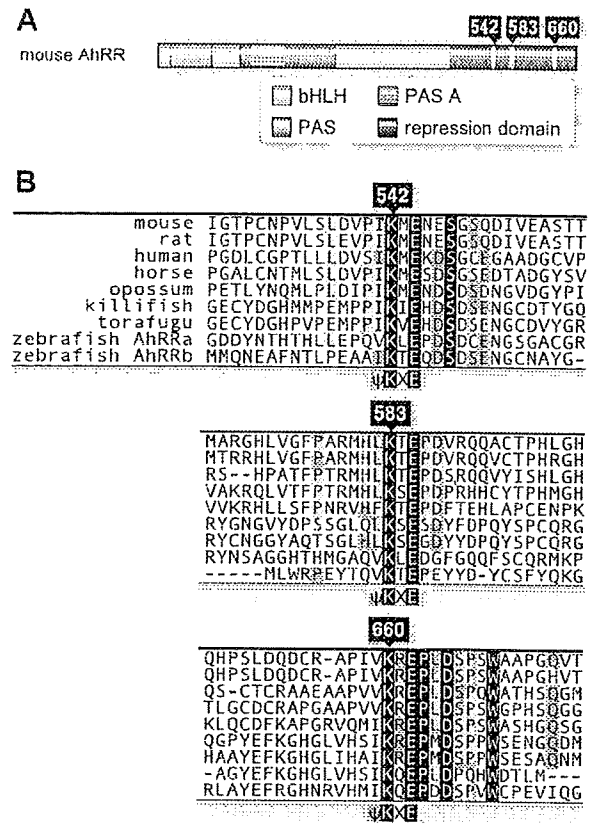


FIGURE 1. **Three conserved SUMOylation sequences in the C-terminal repression domain of AhRR.** *A*, schematic representation of the full-length 701-amino acid mouse AhRR. The characterized domains represented are the basic helix-loop-helix (*bHLH*), Per-Arnt-Sim (*PAS*), and repression domains. Three putative SUMOylation sites are located within the repression domain, with the target lysine residues indicated. *B*, alignments of C-terminal regions of AhRR, including three SUMOylation sites found in mouse, rat, human, horse, opossum, killifish, torafugu, and zebrafish. Conserved amino acids are highlighted in *black*, and the conserved SUMOylation sites and lysine residues are indicated.

published procedure for immunoblot analysis. Immunoblot analysis was performed using anti-FLAG (Sigma), anti-HA (Sigma), anti-SUMO-1 (ALEXIS), anti-Arnt (13), and anti-AhRR antibodies (8).

Fluorescence Analysis—Hepa-1 cells were cultured to subconfluency on coverslips and transfected with expression plasmids for EGFP-AhRR (wild type (WT) or 3KR), His-tagged SUMO-1, and Ubc9, with or without Arnt. After 48 h of transfection, cells were incubated with Hoechst DNA stain, and fluorescent images were observed using fluorescence microscopy (magnification ×1,000).

RESULTS

Three Evolutionarily Conserved SUMOylation Sites in AhRR—To gain insight into the function of AhRR, we inspected the amino acid sequence of mouse AhRR to identify structural motifs for potential covalent modification sites. We found three SUMOylation consensus sequences at amino acid positions 542, 583, and 660 (Fig. 1*A*). All of these sequences are located within the repression domain of AhRR (8). Three SUMOylation sites are well conserved across a broad range of vertebrate species, including mammals and fish (Fig. 1*B*). Other AhRR C-ter-

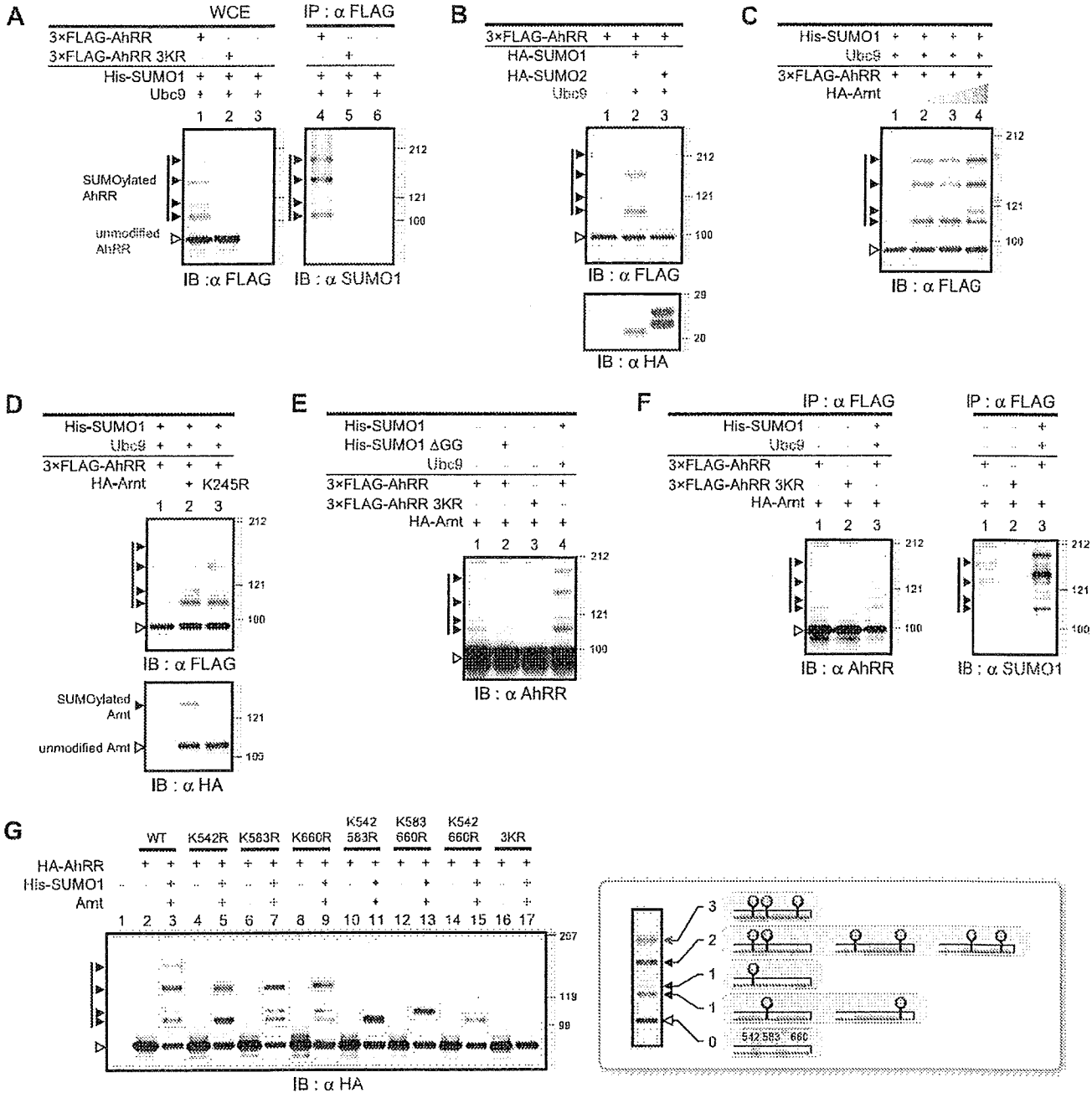
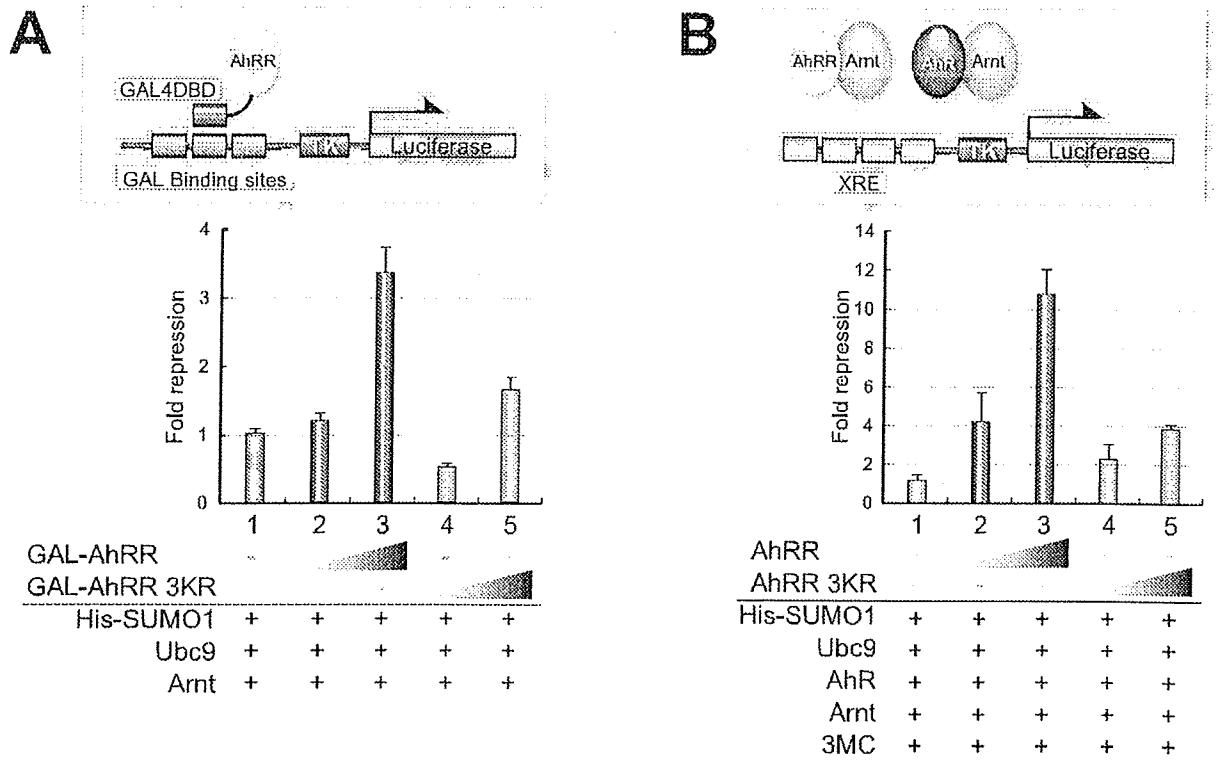
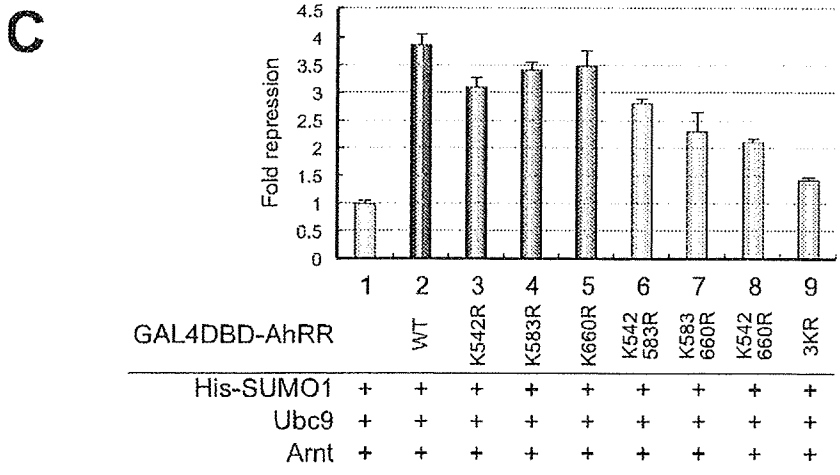


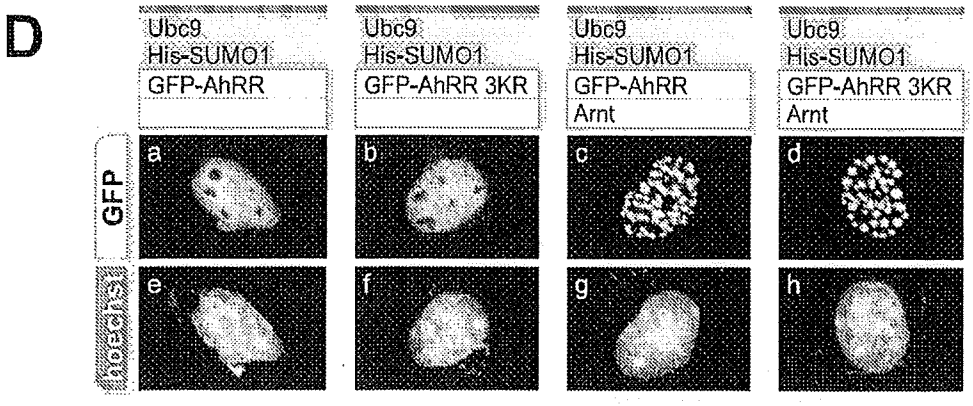
FIGURE 2. **Lysines at 542, 583, and 660 amino acids of AhRR are modified by SUMO-1.** *A* and *F*, COS-7 cells were cotransfected with indicated plasmids, and whole cell extracts (*WCE*) were prepared 48 h after transfection and immunoprecipitated (*IP*) with anti-FLAG antibodies. Immunoprecipitates were analyzed by immunoblotting (*IB*) with the indicated antibodies. Crude lysates were analyzed by immunoblotting to control protein expressions. *B–E* and *G*, COS-7 cells were cotransfected with the indicated expression plasmids. Cell extracts were prepared 48 h after transfection and analyzed by immunoblotting with the indicated antibodies as described above. *Black* and *white arrowheads* indicate SUMOylated or unmodified AhRR, respectively. In *G*, the *right panel* presents the SUMOylation sites and mobility of AhRR.

minal regions have no obvious sequence conservation, suggesting that the putative SUMOylation motifs play an important role in AhRR transcriptional repression activity.

Lysines at Amino Acids 542, 583, and 660 of AhRR Are Modified by SUMO-1—To examine whether these potential SUMO modification sites are actually SUMOylated *in vivo*, we replaced the three conserved lysines with arginines to create the AhRR 3KR mutant. COS-7 cells were cotransfected with expression plasmids for 3xFLAG-AhRR WT or 3xFLAG-AhRR 3KR, along with His-SUMO-1 and Ubc9. In addition to a band for AhRR (~80 kDa), Western blotting using anti-FLAG antibodies detected four bands with slower mobilities when 3xFLAG-AhRR WT was cotransfected, presumably corresponding to various SUMOylated AhRRs (Fig. 2*A*, *left panel*). These bands were also detected without adding Ubc9, although bands were weaker than those in the presence of added Ubc9 (data not shown). In contrast, when 3xFLAG-AhRR 3KR was cotransfected, no slower mobility bands were observed. When cell







extracts were immunoprecipitated with anti-FLAG antibodies, followed by Western blotting using anti-SUMO-1 antibodies, four higher molecular weight bands with the same mobilities were detected (Fig. 2*A*, *right panel*). These results indicate that AhRR is actually SUMOylated *in vivo*, at Lys-542, Lys-583, and Lys-660.

We further examined whether these lysines were also modified by SUMO-2, but when the SUMO-2 plasmid was cotransfected, no modified product of AhRR was observed (Fig. 2*B*). We detected a doublet band of SUMO-2 that was also observed in other reports overexpressing SUMO-2 (14, 15), although the reason is not clear. One possible explanation is that a higher molecular weight band corresponds to immature SUMO-2, which produced the mature SUMO-2 (lower band) by cleaving C-terminal 11 amino acids with SUMO-specific proteases (SENPs) (16). Likewise, addition of any of the PIAS family proteins, which are well characterized SUMO E3 ligases, did not result in increased SUMOylation of AhRR (data not shown). Taken together, these results indicate that AhRR is specifically modified by SUMO-1.

We next investigated whether the heterodimer partner Arnt affects the SUMOylation of AhRR. Arnt is also modified by SUMO-1 (17), and we were interested to know how the SUMOylation of each AhRR and Arnt affects that of the other. As shown in Fig. 2*C*, addition of Arnt significantly enhanced the SUMOylation of AhRR in a dose-dependent manner. Next, to examine whether SUMOylation of Arnt is necessary for enhancement of AhRR SUMOylation, we transfected cells with a plasmid expressing the Arnt K245R mutant plasmid; we observed no difference in AhRR SUMOylation regardless of whether the Arnt WT or K245R mutant was expressed (Fig. 2*D*). These results indicate that heterodimer formation between Arnt and AhRR, but not Arnt SUMOylation, enhances the SUMOylation of AhRR.

Then, we tested whether AhRR was SUMOylated by an endogenous SUMOylation system. When AhRR was expressed in COS-7 cells without exogenous SUMO-1 and Ubc9, four bands, corresponding to the SUMOylated AhRR bands, were also observed (Fig. 2*E*). Bands were of slightly lower mobility than the His-SUMO-1-modified AhRR because of the His tag (Fig. 2*E*, *lanes 1* and *4*). These bands were decreased in intensity when the dominant-negative SUMO-1 mutant with deletion of C-terminal GG residues was cotransfected and disappeared when AhRR 3KR was expressed (Fig. 2*E*, *lanes 2 and 3*). Cell extracts of AhRR-transfected COS-7 cells were immunoprecipitated with anti-FLAG antibodies, followed by Western blotting using anti-SUMO-1 antibodies, and four SUMOylated bands were detected (Fig. 2*F*). These results showed that AhRR was also SUMOylated by the endogenous SUMOylation system.

There are three putative SUMOylation sites in the AhRR amino acid sequence, and four SUMOylated bands were detected in the SUMOylation experiment. We were interested to investigate which sites are actually SUMOylated. We generated arginine mutations in the three lysine residues, either alone or in combination, to identify the SUMOylated bands of AhRR. As shown in Fig. 2*G*, SUMOylated AhRR bands with different mobilities were observed when AhRRs with different Lys-to-Arg mutations were expressed. From the electrophoretic mobilities and the sites of Lys-to-Arg replacement, we were able to attribute the bands to the specifically SUMOylated AhRR species shown in Fig. 2*G*. Taken together, these results showed that AhRR was mono-, di-, and tri-SUMOylated on Lys-542, Lys-583, and Lys-660, and all of these SUMOylations were significantly enhanced when Arnt was coexpressed with AhRR.

SUMO Modification of AhRR Is Important for Its Efficient Transcriptional Repression Activity—Because SUMOylation usually increases the activity of transcriptional repressors, we next examined the effects of SUMOylation on the function of AhRR as a transcriptional repressor. First, we performed a reporter assay using the luciferase gene under the control of the thymidine kinase promoter ligated with GAL4-binding sites (3xGAL-TK-Luc). Transcriptional repression activity of AhRR fused with the GAL4 DNA-binding domain (GAL4DBD) was assessed by measuring the luciferase gene expression driven by the thymidine kinase promoter. AhRR WT repressed the luciferase activity dose-dependently, whereas the 3KR mutant of AhRR significantly reduced the repressive activity compared with the WT (Fig. 3*A*). Expression level of AhRR WT and 3KR were normalized using Western blot analysis (data not shown). Next, we used a luciferase reporter gene driven by four tandemly repeated XREs to assess the repression activity of AhRR. AhRR WT repressed the luciferase expression driven by the XRE sequence to a greater degree than AhRR 3KR (Fig. 3*B*). For Fig. 3 (*A* and *B*), essentially the same results were also observed when SUMO-1 and Ubc9 were not exogenously expressed using HeLa cells (supplemental Fig. S1, *A* and *B*, *lanes 1–3*), indicating that endogenous SUMOylation activity is sufficient for AhRR repression activity. These results indicate that the SUMOylation of AhRR is important for its transcriptional repression activity.

To determine the contribution of each of the three lysines to the repressive activity of AhRR, the GAL4 reporter assay was performed using the mutant AhRR-GAL4DBD fusion genes with the Lys-to-Arg mutations in all three lysine positions,

FIGURE 3. **SUMO modification sites of AhRR and their effects on its transcriptional repression activity.** *A*, shown is the transcriptional repression activity of AhRR WT and 3KR. Hepa-1 cells were transiently transfected with the expression plasmids for GAL4DBD-AhRR WT or 3KR, His-tagged SUMO-1, Ubc9, Arnt, and GAL-TK-Luc reporter gene containing three GAL-binding sites. Cell extracts were prepared 48 h after transfection and used for luciferase assays. The -fold repression is relative to the reporter gene alone. *B*, HeLa cells were transiently transfected with the expression plasmids for AhRR WT or 3KR, His-tagged SUMO-1, Ubc9, AhR, Arnt, and the 4xXRE-TK-Luc reporter gene containing four XREs. After 48 h of transfection, cells were treated with 2 μM of 3-MC or Me_2SO; 24 h later, cell extracts were prepared and used for luciferase assays. *C*, Hepa-1 cells were transiently transfected with the expression plasmids for GAL4DBD-AhRR WT; 3KR or AhRR with Lys-to-Arg mutation(s) of each of the three lysines, either alone or in combination; His-tagged SUMO-1, Ubc9, Arnt, and GAL-TK-Luc reporter gene containing three GAL-binding sites. Cell extracts were prepared 48 h after transfection and used for luciferase assays. *D*, the subnuclear distribution of EGFP-AhRR is not changed in the SUMOylation mutant. Hepa-1 cells were transfected with expression plasmids of EGFP-AhRR WT or 3KR, His-tagged SUMO-1, and Ubc9, with or without Arnt. After 48 h of transfection, cells were incubated with Hoechst DNA stain (*lower panels*), and fluorescence images were taken (*upper panels*).

either alone or in combination. As shown in Fig. 3*C*, the SUMOylation of each lysine residue contributes similarly to the repression activity of AhRR. These results indicate that SUMO-1 modification of Lys-542, Lys-583, and Lys-660 more or less contribute equally to the transcriptional repression activity of AhRR.

SUMOylation of AhRR Is Required for Interaction of AhRR with Corepressor Components—SUMO modification often regulates the subcellular localization of the target proteins. To examine whether SUMO modification affects nuclear localization of AhRR, we transfected Hepa-1 cells with EGFP-AhRR WT or EGFP-AhRR 3KR and observed their subcellular distributions using fluorescence microscopy. Both EGFP-AhRR WT and EGFP-AhRR 3KR gave a similar diffuse nuclear localization pattern (Fig. 3*D*, *panels a* and *b*). In contrast, coexpression of Arnt markedly changed the subcellular localization of both proteins to a speckled distribution pattern (Fig. 3*D*, *panels c* and *d*), with fewer than 40% of the transfected cells retaining a diffuse pattern. This speckled localization pattern is consistent with the Arnt localization pattern (17). The same results were also observed when SUMO-1 and Ubc9 were not exogenously expressed (data not shown). These results indicate that SUMO-1 modification of AhRR does not affect subcellular localization of AhRR, whereas Arnt alters the subnuclear localization of AhRR to the speckled pattern. SUMO modification is also known to play a key role in protein-protein interaction. Previously, we demonstrated that the C-terminal repression domain, which carries the three SUMO-1 modified lysines, interacts with ANKRA2, HDAC4, and HDAC5; these interactions are important for the repression activity of AhRR. Therefore, we examined whether the SUMOylation of AhRR affects the interaction with ANKRA2, HDAC4, and HDAC5. COS-7 cells were transfected with the HA-tagged AhRR WT or 3KR mutant, along with FLAG-tagged ANKRA2, HDAC4, or HDAC5. The cell extracts prepared from the transfected cells were immunoprecipitated with an anti-HA antibody. ANKRA2 was co-immunoprecipitated with AhRR WT, and HDAC4 was coprecipitated only when ANKRA2 was coexpressed (Fig. 4*A*, *left* and *right panels*, *lanes 2* and *4*), consistent with the previous report (8). However, when the HA-tagged mutant AhRR 3KR was transfected instead of AhRR WT, immunoprecipitates with an anti-HA antibody detected only a small amount of ANKRA2 and HDAC4 (Fig. 4, *A*, *left* and *right panels*, *lane 3*, and *C*), indicating that SUMOylation of AhRR is necessary for efficient interaction with ANKRA2 and HDAC4. As expected, HDAC5 was co-immunoprecipitated with AhRR WT even in the absence of ANKRA2 (Fig. 4*B*, *left and right panels*, *lanes 2* and *4*), but binding of HDAC5 with AhRR 3KR was much reduced (Fig. 4*B*, *left* and *right panels*, *lane 3*, and *C*). Taken together, these results show that the SUMOylation of AhRR is necessary for efficient interaction with ANKRA2, HDAC4, and HDAC5, resulting in its increased transcriptional repression activity.

AhRR, but Not AhR, Enhances SUMO-1 Modification of Arnt—Arnt forms a heterodimer with AhRR, colocalizes with AhRR in the nucleus, and enhances SUMOylation of AhRR (Figs. 2*C* and 3*D*). Arnt was previously reported to be SUMOylated, and SUMOylation of Arnt suppresses GAL4DBD-Arnt-mediated transactivation through dissociation from PML (17). Next, we

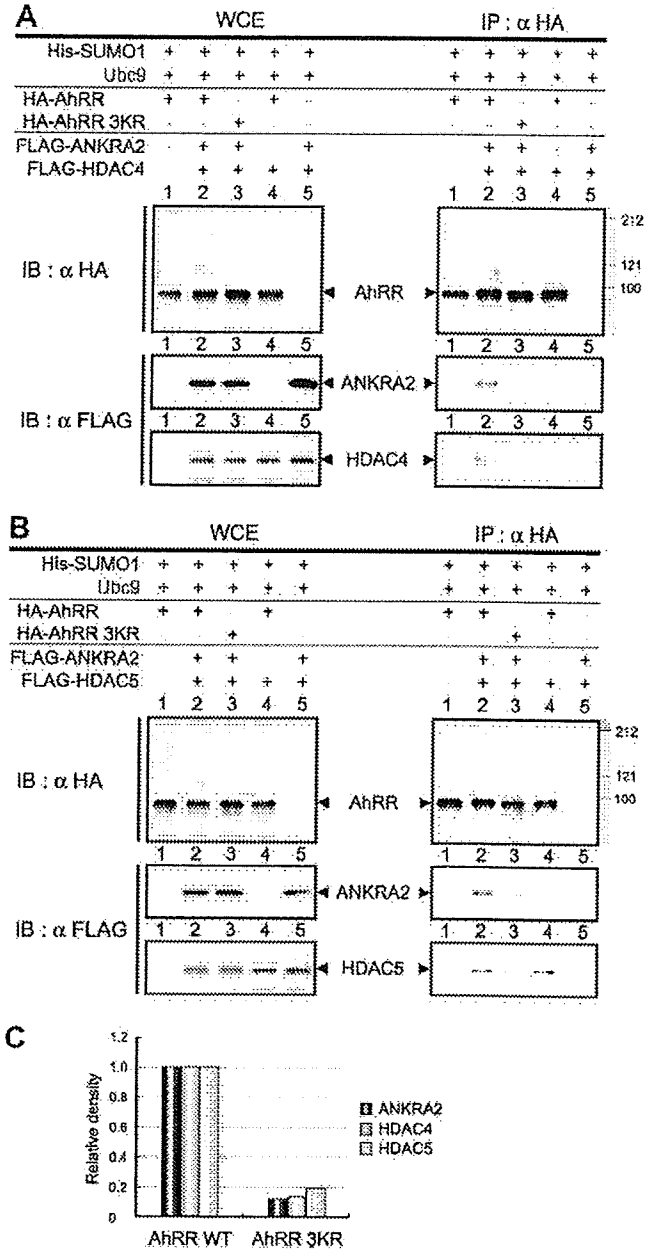


FIGURE 4. **Interaction of SUMO-1-modified AhRR with ANKRA2, HDAC4, and HDAC5.** *A* and *B*, COS-7 cells were cotransfected with expression plasmids for HA-tagged AhRR WT or 3KR, His-tagged SUMO-1, and Ubc9, with or without FLAG-tagged ANKRA2 and FLAG-tagged HDAC4 for *A*, or FLAG-tagged HDAC5 for *B*. Whole cell extracts (*WCE*) were prepared 48 h after transfection and immunoprecipitated (*IP*) with anti-HA antibodies. Immunoprecipitates were analyzed by immunoblot (*IB*) with the indicated antibodies (*B*, *right panels*). Crude lysates were analyzed by immunoblot to control for protein expression (*B*, *left panels*). *C*, the relative densities of the ANKRA2, HDAC4, and HDAC5 bands, which were co-immunoprecipitated with either AhRR WT or 3KR, were calculated with NIH Image software. Values are relative to AhRR WT.

examined whether AhRR or AhR affects the SUMO modification of Arnt. Plasmid 3xFLAG-tagged Arnt was transiently expressed in COS-7 cells, together with HA-tagged AhRR or HA-tagged AhR, and the SUMOylated Arnt was examined by Western blotting using an anti-Arnt antibody. Although SUMO-1-modified Arnt was barely detected in the absence of AhRR, SUMO-1-modified Arnt was significantly increased in a

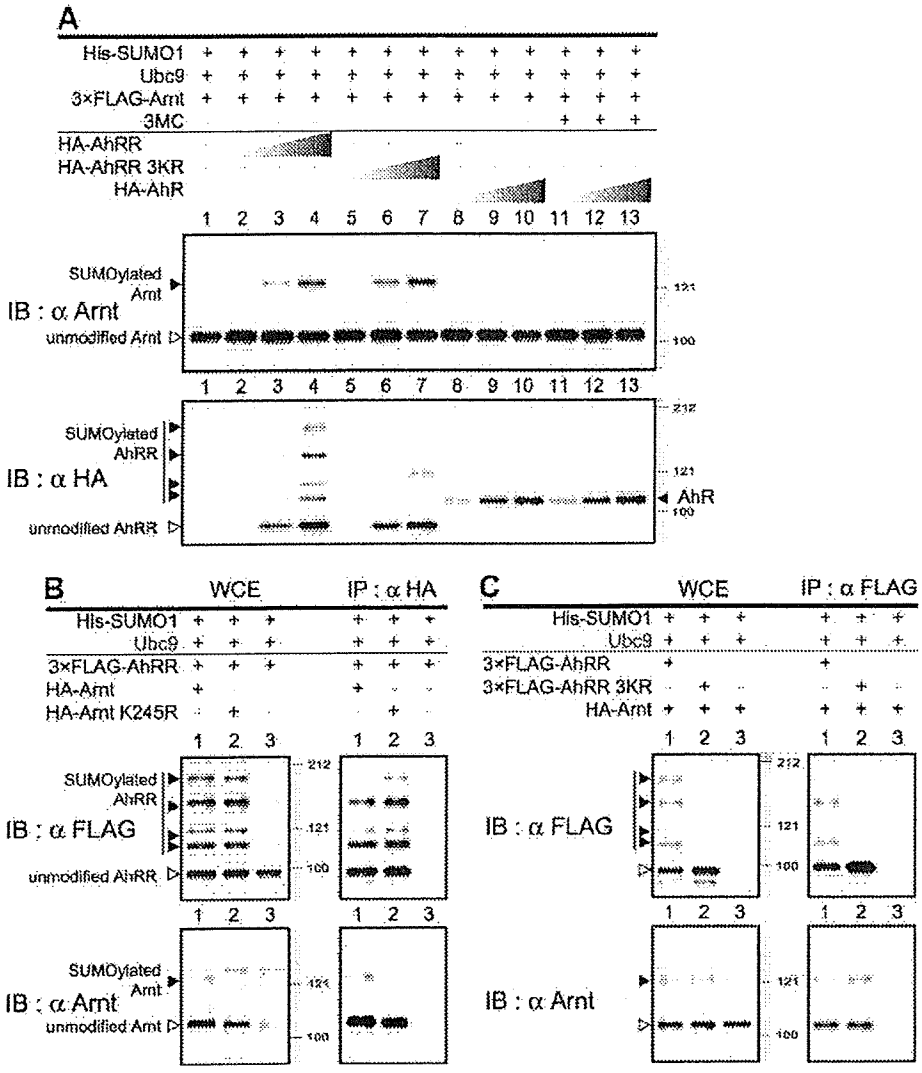


FIGURE 5. **AhRR, but not AhR, and Arnt mutually stimulate one another's SUMO-1 modification.** *A*, COS-7 cells were cotransfected with expression plasmids for HA-tagged AhRR WT or 3KR or HA-tagged AhR, 3xFLAG-tagged Arnt, His-tagged SUMO-1, and Ubc9. After 48 h of transfection, cells for *lanes 11–13* were treated with 2 μM of 3-MC or Me_2SO; 24 h later, whole cell extracts (*WCE*) were prepared and analyzed by immunoblot (*IB*) using the indicated antibodies. *B and C*, COS-7 cells were cotransfected with the indicated expression plasmids. Cell extracts were prepared 48 h after transfection and immunoprecipitated (*IP*) with the indicated antibodies. Immunoprecipitates were analyzed by immunoblot with the indicated antibodies. Crude lysates were analyzed by immunoblot to control for protein expression. *Black* and *white arrowheads* indicate SUMOylated or unmodified AhRR, respectively.

dose-dependent manner upon addition of AhRR (Fig. 5*A*, *lanes 1–4*). The same enhancing effect was observed when AhRR 3KR was cotransfected (Fig. 5*A*, *lanes 5–7*). Therefore, regardless of its SUMOylation, AhRR is necessary for enhancement of Arnt SUMOylation. AhR is also a partner molecule of Arnt for heterodimer formation. We were interested in examining how AhR affects the SUMO modification of Arnt. When HA-tagged AhR was coexpressed with Arnt, no enhancement in the SUMOylation of Arnt was observed, even when the ligands of AhR were added (Fig. 5*A*, *lanes 8–13*). Thus, AhRR and Arnt mutually enhance the SUMOylation of their partner molecules, which may result in enhancement of the repression activity of the AhRR/Arnt heterodimer.

At the same time, these observations raised the question of whether these SUMOylations affect the interaction between AhRR and Arnt. To address this question, COS-7 cells were transfected with expression plasmids for 3xFLAG-tagged AhRR or 3xFLAG-tagged AhRR 3KR, together with HA-tagged Arnt WT or HA-tagged Arnt K245R. Cell extracts were prepared from the transfected cells and immunoprecipitated with anti-HA or anti-FLAG antibodies. No effect of SUMOylation was observed on the interaction between AhRR and Arnt (Fig. 5, *B* and *C*, *lanes 1* and *2*).

SUMOylation of Both AhRR and Arnt Is Important for Repressor Activity of the AhRR·Arnt Complex—We next assessed the potential effects of the SUMOylated Arnt on the repressor activity of the AhRR/Arnt heterodimer. Hepa-1 cells were transfected with expression plasmids for GAL4DBD-fused Arnt WT or K245R, together with His-SUMO-1 and Ubc9, and either of AhR, AhRR WT, or 3KR. GAL4DBD-Arnt WT showed an autonomous transactivation activity, and addition of AhR enhanced this transactivation (Fig. 6*A*, *lanes 1–3*). When non-SUMOylation mutant Arnt K245R was added, there was no difference in transactivation activity from Arnt WT, in agreement with the observation in Fig. 5*A* that Arnt was barely SUMOylated under these conditions (Fig. 6*A*, *lanes 2–5*). This result is also consistent with the previous report that the SUMOylation of Arnt did not affect the transactivation activity of AhR/Arnt or Hif-1α/Arnt (17). When we added AhRR WT, the luciferase expression was remarkably reduced, whereas the addition of AhRR 3KR inhibited the expression of luciferase activity to a lesser extent, with the residual luciferase activity significantly higher than with the wild type AhRR (Fig. 6*A*, *lanes 6* and *7*). This tendency was more remarkable when both non-SUMOylation mutants of AhRR and Arnt were used (Fig. 6*A*, *lane 9*). Next, to further assess the importance of SUMOylation of the AhRR/Arnt heterodimer for its repressor activity, we used a luciferase reporter gene driven by four tandemly repeated XREs. As shown in Fig. 6*B*, the result was almost the same as that as shown in Fig. 6*A*. These experiments were also performed without added SUMO-1 and Ubc9 using HeLa cells, which showed essentially

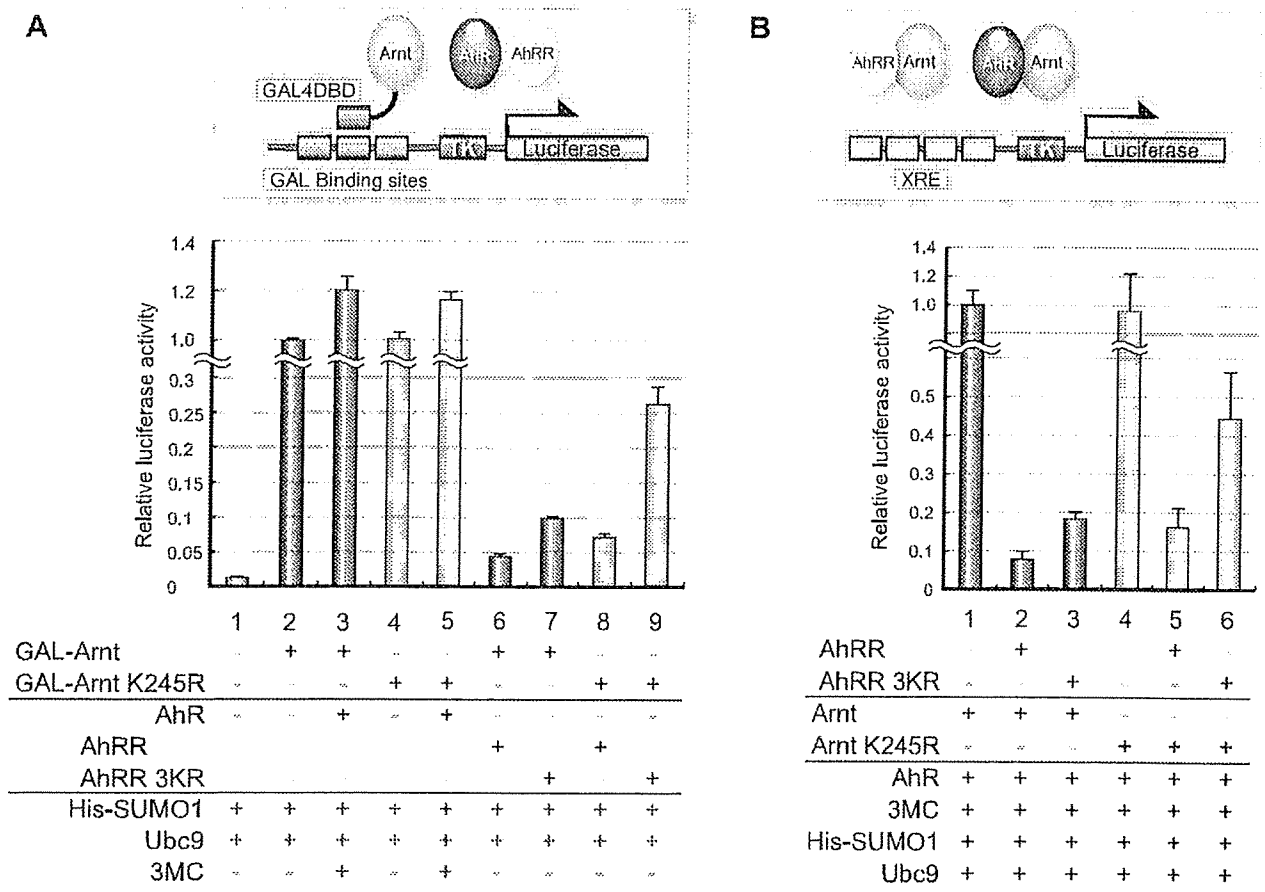


FIGURE 6. **SUMOylation of both AhRR and Arnt is important for the repression activity of the AhRR·Arnt complex.** SUMOylation sites of both AhRR and Arnt are important for the transcriptional repression activity of the AhRR·Arnt complex. *A*, Hepa-1 cells were transiently transfected with the expression plasmids for GAL4DBD-Arnt WT or K245R, AhRR WT or 3KR or AhR, His-tagged SUMO-1, Ubc9, and the GAL-TK-Luc reporter gene containing three GAL-binding sites. After 48 h of transfection, cells for *lanes 3* and *5* were treated with 2 μM of 3-MC or Me_2SO; 24 h later, cell extracts were used for luciferase assays. Luciferase activities are shown as values relative to *lane 2*. *B*, COS-7 cells were transiently transfected with the expression plasmids for Arnt WT or K245R, AhRR WT, 3KR, or AhR, His-tagged SUMO-1, Ubc9, and 4xXRE-TK-Luc reporter gene containing four XRE sequences. After 48 h of transfection, cells were treated with 2 μM of 3-MC or Me_2SO; 24 h later, cell extracts were prepared and used for luciferase assays. Luciferase activities are shown as values relative to *lane 1*.

the same results (supplemental Fig. S1*B*). Taken together, these results show that SUMOylation of both AhRR and Arnt is important for the efficient repressor activity of the AhRR/Arnt heterodimer (Fig. 7).

DISCUSSION

When amino acid sequences of the C-terminal region of AhRR were compared among mammals and fish, three SUMOylation sites including minimal adjacent sequences were revealed to be distinctly conserved (Fig. 1*B*), suggesting that these sequences are involved in some fundamental biological processes. A newly reported composite motif named PDSM (phosphorylation-dependent SUMOylation motif), which contains the SUMO consensus sequence with an adjacent proline-directed phosphorylation site (ψ*KXEXXX*SP) (18–23), undergoes phosphorylation-dependent SUMOylation in many transcription factors, including heat shock proteins (HSPs), peroxisome proliferator-activated receptor γ, MEF2, and GATA-1. Accelerated SUMOylation of these factors by phosphorylation enhances the repression of transcriptional activity. The SUMOylation site at Lys-660 in mouse AhRR, which is well conserved across species, seems to resemble the SUMO consensus sequence accompanying the proline-directed phosphorylation (ψ*KXEXXX*SP), but its functional analysis remains to be seen.

All of the conserved SUMOylation sites of AhRR were found to be SUMOylated more or less equally in our *in vivo* cell culture system. These SUMOylations in AhRR were significantly enhanced by the presence of Arnt, a partner molecule of the AhRR/Arnt heterodimer. Likewise, Arnt was reported previously to be SUMOylated at Lys-245 (17); this SUMOylation was also stimulated by AhRR, but not by AhR, another partner molecule of the transcription-active heterodimer AhR/Anrt. Thus, one of the partner molecules of the AhRR·Arnt repressor complex mutually enhances the SUMOylation of the other (Figs. 2*C* and 5*A*). The PIAS proteins are members of a well characterized SUMO E3 ligase family, consisting of PIAS1, PIASxα, PIASxβ, PIASγ, and PIAS3. However, none of the family members added into the cultured cells could enhance the SUMOylation of AhRR (data not shown). Unlike ubiquitin E3 ligases, apparently structurally unrelated proteins can serve as SUMO E3 ligases, providing a scaffold bridging between Ubc9 and

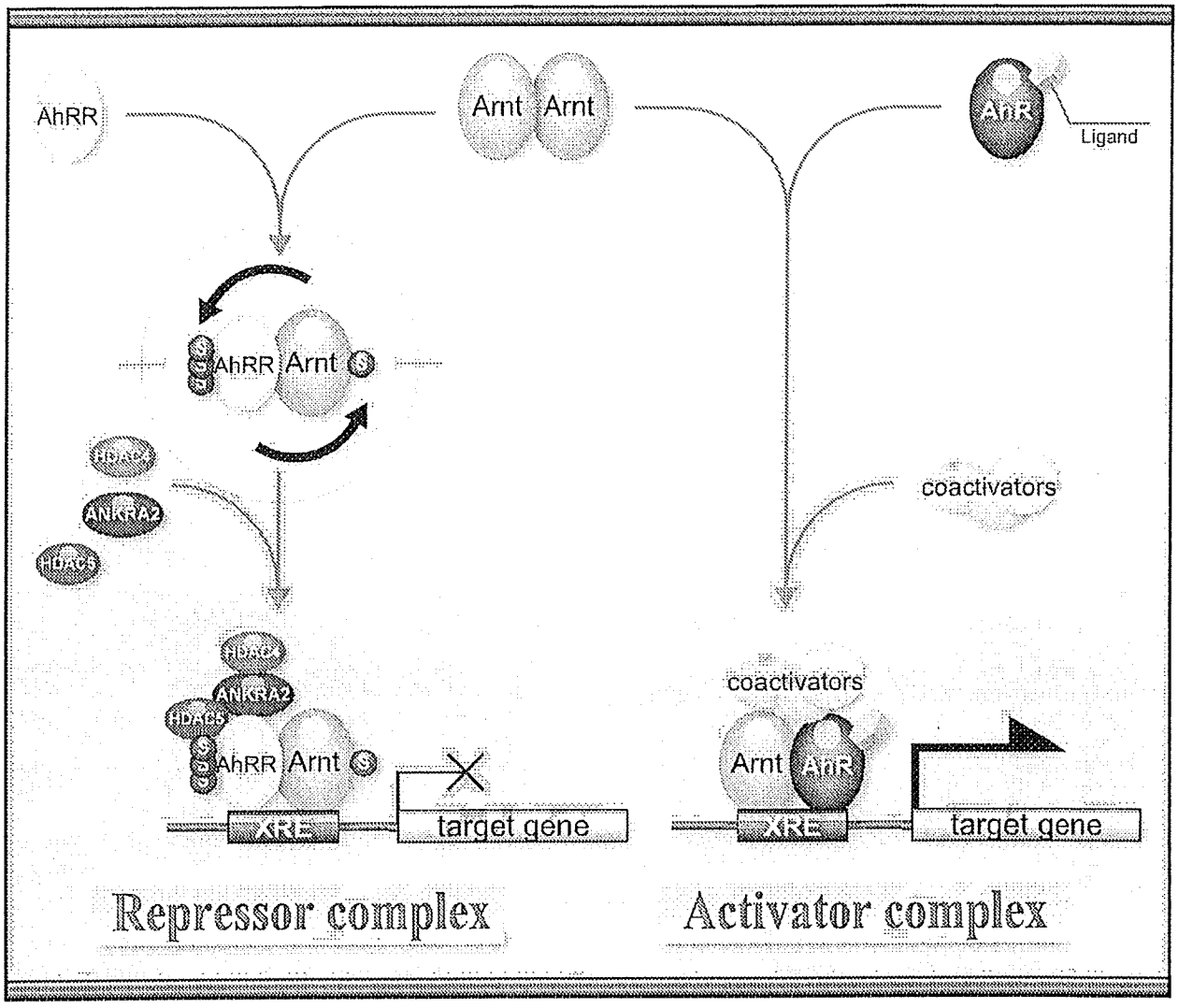


FIGURE 7. **Proposed model for the transcriptional regulation mechanism of the AhR/Arnt activator complex and the AhRR/Arnt repressor complex.** Unmodified Arnt forms a heterodimer with AhR and recruits coactivators such as CBP/p300 to form the transcriptional activator complex. Meanwhile, Arnt forms a heterodimer with AhRR, which significantly enhances SUMOylation of both proteins. SUMOylated AhRR recruits corepressors ANKRA2, HDAC4, and HDAC5 to form the transcriptional repressor complex.

SUMOylation substrates. Whereas the PIAS family apparently plays no role in AhRR modification, Arnt could significantly enhance SUMOylation of AhRR, and the reverse is true with respect to Arnt SUMOylation. Because both AhRR (data not shown) and Arnt (17) interact with Ubc9 and their partner molecules, SUMOylation substrates, it is reasonable to consider that AhRR and Arnt serve as the SUMO E3 ligase to each other. This could be substantiated experimentally by *in vitro* reconstitution of SUMOylation. Interestingly, it has recently been reported that AhR is able to act as an E3 ubiquitin ligase to estrogen receptors and androgen receptors in a ligand-dependent manner (24), indicating the potential role of bHLH-PAS proteins, including AhR, to be able to display an E3 ligase activity with a ubiquitin family protein, in addition to its transcriptional activity.

In the nucleus, unmodified Arnt interacts and colocalizes with PML, which enhances the transactivation activity of Arnt; in contrast, SUMOylated Arnt dissociates from PML, resulting in the suppression of Arnt transcription activity in the GAL4DBD-Arnt-mediated transactivation assay (17). In contrast to AhRR, SUMOylation of Arnt is enhanced by PIAS1, and a substantial amount of Arnt is present in the nucleus in SUMOylated form (17). When AhRR is synthesized and transported into the nucleus through the NLS signal, AhRR may form a heterodimer with Arnt or SUMOylated Arnt, resulting in the enhanced SUMOylation of AhRR in the heterodimer complex. Thus, the SUMOylated AhRR and Arnt complex becomes competent for recruitment of corepressor components such as ANKRA2, HDAC4, and HDAC5. It remains to be investigated whether this series of transcription suppressor complexes are formed directly on the XRE sequence of the target genes or in the nucleoplasm prior to DNA binding. Our previous data showed that treatment of ANKRA2 small interfering RNA resulted in reduction of the AhRR-mediated repression of *Cyp1a1* mRNA expression under normal conditions (8). Because transcriptional inhibition by the AhRR·Arnt

complex is reversed by trichostatin A, an inhibitor of histone deacetylase (8), not only competitive binding to the XRE sequence, but also the HDAC activity of the AhRR/Arnt heterodimer is involved in repression by the AhRR/Arnt heterodimer. In Figs. 3 and 6, AhRR 3KR also showed some repression activity even if all SUMOylation sites are mutated, which could be explained by the residual interaction of AhRR 3KR with ANKRA2, HDAC4, and HDAC5 or competitive binding mechanisms for the XRE sequence with the AhR/Arnt heterodimer (Figs. 3*B* and 6*B*).

In this study, we have revealed a novel mechanism of transcriptional repression by the AhRR/Arnt heterodimer involving SUMO-1 modification. Further studies are required to reveal the detailed molecular mechanisms by which AhRR and Arnt are SUMOylated and subsequently recruit the corepressor components, as well as the temporal relationship between these events and the switching on/off of the target genes.

Acknowledgment—We thank Y. Nemoto for clerical work.

REFERENCES

1. Mimura, J., Ema, M., Sogawa, K., and Fujii-Kuriyama, Y. (1999) *Genes Dev.* **13,** 20–25
2. Baba, T., Mimura, J., Gradin, K., Kuroiwa, A., Watanabe, T., Matsuda, Y., Inazawa, J., Sogawa, K., and Fujii-Kuriyama, Y. (2001) *J. Biol. Chem.* **276,** 33101–33110
3. Karchner, S. I., Franks, D. G., Powell, W. H., and Hahn, M. E. (2002) *J. Biol. Chem.* **277,** 6949–6959
4. Nishihashi, H., Kanno, Y., Tomuro, K., Nakahama, T., and Inouye, Y. (2006) *Biol. Pharm. Bull.* **29,** 640–647
5. Hosoya, T., Harada, N., Mimura, J., Motohashi, H., Takahashi, S., Nakajima, O., Morita, M., Kawauchi, S., Yamamoto, M., and Fujii-Kuriyama, Y. (2008) *Biochem. Biophys. Res. Commun.* **365,** 562–567
6. Zudaire, E., Cuesta, N., Murty, V., Woodson, K., Adams, L., Gonzalez, N., Martinez, A., Narayan, G., Kirsch, I., Franklin, W., Hirsch, F., Birrer, M., and Cuttitta, F. (2008) *J. Clin. Investig.* **118,** 640–650
7. Kanno, Y., Takane, Y., Izawa, T., Nakahama, T., and Inouye, Y. (2006) *Biol. Pharm. Bull.* **29,** 1254–1257
8. Oshima, M., Mimura, J., Yamamoto, M., and Fujii-Kuriyama, Y. (2007) *Biochem. Biophys. Res. Commun.* **364,** 276–282
9. Hay, R. T. (2005) *Mol. Cell* **18,** 1–12
10. Meulmeester, E., and Melchior, F. (2008) *Nature* **452,** 709–711
11. Mizushima, S., and Nagata, S. (1990) *Nucleic Acids Res.* **18,** 5322
12. Sekine, H., Mimura, J., Yamamoto, M., and Fujii-Kuriyama, Y. (2006) *J. Biol. Chem.* **281,** 37507–37516
13. Sogawa, K., Nakano, R., Kobayashi, A., Kikuchi, Y., Ohe, N., Matsushita, N., and Fujii-Kuriyama, Y. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 1936–1940
14. Gong, L., and Yeh, E. T. (2006) *J. Biol. Chem.* **281,** 15869–15877
15. Motohashi, H., Katsuoka, F., Miyoshi, C., Uchimura, Y., Saitoh, H., Francastel, C., Engel, J. D., and Yamamoto, M. (2006) *Mol. Cell. Biol.* **26,** 4652–4663
16. Johnson, E. S. (2004) *Annu. Rev. Biochem.* **73,** 355–382
17. Tojo, M., Matsuzaki, K., Minami, T., Honda, Y., Yasuda, H., Chiba, T., Saya, H., Fujii-Kuriyama, Y., and Nakao, M. (2002) *J. Biol. Chem.* **277,** 46576–46585
18. Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A., and Sistonen, L. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 45–50
19. Yang, X. J., and Gregoire, S. (2006) *Mol. Cell* **23,** 779–786
20. Yamashita, D., Yamaguchi, T., Shimizu, M., Nakata, N., Hirose, F., and Osumi, T. (2004) *Genes Cells* **9,** 1017–1029
21. Gregoire, S., and Yang, X. J. (2005) *Mol. Cell. Biol.* **25,** 2273–2287
22. Kang, J., Gocke, C. B., and Yu, H. (2006) *BMC Biochem.* **7,** 5
23. Shalizi, A., Gaudilliere, B., Yuan, Z., Stegmuller, J., Shirogane, T., Ge, Q., Tan, Y., Schulman, B., Harper, J. W., and Bonni, A. (2006) *Science* **311,** 1012–1017
24. Ohtake, F., Baba, A., Takada, I., Okada, M., Iwasaki, K., Miki, H., Takahashi, S., Kouzmenko, A., Nohara, K., Chiba, T., Fujii-Kuriyama, Y., and Kato, S. (2007) *Nature* **446,** 562–566

Structure-Activity–Dependent Regulation of Cell Communication by Perfluorinated Fatty Acids using *in Vivo* and *in Vitro* Model Systems

Brad L. Upham,[1] Joon-Suk Park,[1] Pavel Babica,[1] Iva Sovadinova,[1] Alisa M. Rummel,[1] James E. Trosko,[1] Akihiko Hirose,[2] Ryuichi Hasegawa,[2] Jun Kanno,[3] and Kimie Sai[3]**

[1]Department of Pediatrics and Human Development, National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan, USA; [2]Division of Risk Assessment, and [3]Division of Cellular and Molecular Toxicology, National Institute of Health Sciences, Tokyo, Japan

BACKGROUND: Perfluoroalkanoates, [e.g., perfluorooctanoate (PFOA)], are known peroxisome proliferators that induce hepatomegaly and hepatocarcinogenesis in rodents, and are classic nongenotoxic carcinogens that inhibit *in vitro* gap-junctional intercellular communication (GJIC). This inhibition of GJIC is known to be a function of perfluorinated carbon lengths ranging from 7 to 10.

OBJECTIVES: The aim of this study was to determine if the inhibition of GJIC by PFOA but not perfluoropentanoate (PFPeA) observed in F344 rat liver cells *in vitro* also occurs in F344 rats *in vivo* and to determine mechanisms of PFOA dysregulation of GJIC using *in vitro* assay systems.

METHODS: We used an incision load/dye transfer technique to assess GJIC in livers of rats exposed to PFOA and PFPeA. We used *in vitro* assays with inhibitors of cell signaling enzymes and antioxidants known to regulate GJIC to identify which enzymes regulated PFOA-induced inhibition of GJIC.

RESULTS: PFOA inhibited GJIC and induced hepatomegaly in rat livers, whereas PFPeA had no effect on either end point. Serum biochemistry of liver enzymes indicated no cytotoxic response to these compounds. *In vitro* analysis of mitogen-activated protein kinase (MAPK) indicated that PFOA, but not PFPeA, can activate the extracellular receptor kinase (ERK). Inhibition of GJIC, *in vitro*, by PFOA depended on the activation of both ERK and phosphatidylcholine-specific phospholipase C (PC-PLC) in the dysregulation of GJIC in an oxidative-dependent mechanism.

CONCLUSIONS: The *in vitro* analysis of GJIC, an epigenetic marker of tumor promoters, can also predict the *in vivo* activity of PFOA, which dysregulated GJIC via ERK and PC-PLC.

KEY WORDS: extracellular receptor kinase, gap-junctional intercellular communication, mitogen-activated protein kinase, perfluorooctanoate, perfluoropentanoate, phosphatidylcholine-specific-phospholipase C, tumor promotion. *Environ Health Perspect* 117:545–551 (2009). doi:10.1289/ehp.11728 available via *http://dx.doi.org/* [Online 23 October 2008]

Research on the environmental fate and toxicology of halogenated compounds has focused primarily on brominated and chlorinated organics, whereas fluorinated organics received less attention, partly because of the perception that these compounds, which are quite chemically inert, were also biologically inert (Key et al. 1997). However, perfluorinated fatty acids (PFFAs), such as perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS), are found in the environment and have been detected in the blood of animals throughout the world, including the seals of remote arctic regions, indicating widespread distribution (Kannan 2001; Tao 2006; Van de Vijver 2005). Significant levels of PFOA and PFOS have also been detected in the serum of humans, but there is evidence of a significant decline in body burdens of PFOS and PFOA over the last 5–10 years (Calafat et al. 2007). The values from the first National Health and Nutrition Examination Survey (NHANES) conducted from 1999 to 2000 reported geometric means of 30.4 μg PFOS/L and 5.4 μg PFOA/L, and the second NHANES conducted between 2003 and 2004 reported geometric means of 20.7 μg PFOS/L and 3.9 μg PFOA/L (Calafat et al. 2007). Contamination of the environment is not limited to PFOA and PFOS but also includes short-chain perfluorinated alkanoates, such as perfluorobutyrate, perfluoropentanoate (PFPeA), perfluorohexanoate, and perfluoroheptanoate (Skutlarek et al. 2006).

The acute toxicities of PFOA and PFOS in rodent systems are low (Hekster 2003; Kudo and Kawashima 2003). After the absorption of PFOA into the body, it is predominantly distributed in the liver and plasma and, to a lesser extent, the kidney and lungs (Kudo and Kawashima 2003). Thus, the chronic and short-term effects of PFOA in rats are found largely in the liver (Kennedy et al. 2004) and immune system (DeWitt et al. 2008). Peroxisome proliferation in rodent livers is one of the major responses to PFOA, along with subsequent interferences with normal metabolism of fatty acids and cholesterol, and the induction of hepatocellular hypertrophy (Kennedy et al. 2004). Peroxisome-proliferating chemicals are classic nongenotoxic tumor promoters in rodent liver tissue (Cattley et al. 1995), and like other peroxisome proliferators, PFOA has also been shown to strongly promote tumors in rodent livers (Abdellatif et al. 1991). However, peroxisome-proliferating compounds might not be strong tumor promoters in human livers because of species differences in the response to peroxisome proliferators *in vivo*, with rodents more responsive than primates (Klaunig et al. 2003).

Although the underlying mechanisms of tumor promotion might vary, such as the induction of peroxisome proliferation, tumorigenic cells have long been characterized as cells that lose their ability to regulate growth through contact inhibition (Borek and Sachs 1966) and lack the ability to terminally differentiate (Potter 1978), which implies a breakdown in one of the communicating mechanisms (Trosko and Upham 2005). Tumorigenic cells can be benign, leading to the compression of surrounding tissues, or have the potential to acquire genetic mutations that lead to a malignant state where the cancerous cells can invade surrounding tissues. Alteration of cell-to-cell communication via gap junctions has been implicated in the tumorigenic process and is supported by considerable evidence (Trosko and Ruch 2002).

Inhibition of gap-junctional intercellular communication (GJIC) appears to be a necessary, albeit insufficient, step of tumorigenesis and is therefore a common response of cells to tumor promoters, oncogenes, growth factors, and nongenotoxic carcinogens such as peroxisome proliferators (Trosko and Ruch 1998; Trosko and Upham 2005). Although GJIC is modulated by multiple signaling pathways, simple bioassays of intercellular communication can be used to assess dysregulation of gap junctions regardless of the upstream effectors.

Address correspondence to B.L. Upham, Michigan State University, 243 National Food Safety and Toxicology Center, East Lansing, MI 48824 USA. Telephone: (517) 884-2051. Fax: (517) 432-6340. E-mail: upham@msu.edu

*Current address: Division of Medical Safety Science, National Institute of Health Sciences, Tokyo, Japan.

**Current address: Division of Functional Biochemistry and Genomics, National Institute of Health Sciences, Tokyo, Japan.

This research was supported by National Institute of Environmental Health Sciences (NIEHS) grant R01 ES013268-01A2 to B.L.U. and by a Grant-in-Aid for Science Research from the Ministry of Education, Science, Sports, and Culture of Japan (11694334).

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS.

The authors declare they have no competing financial interests.

Received 23 May 2008; accepted 23 October 2008.

Thus, GJIC is an excellent biomarker first to assess the potential tumorigenicity of chemicals and then to use as a cell signaling end point to determine the early molecular events induced by these chemicals.

Cell proliferative diseases, such as cancer, not only require the release of a quiescent cell from growth suppression via down-regulation of GJIC and/or changes in extracellular components (i.e., integrins), but also need to activate mitogenic signaling pathways. The mitogen-activated protein kinase (MAPK) pathways are the major intracellular signaling mechanisms by which a cell activates, via phosphorylation, transcription factors involved in mitogenesis (Denhardt 1996). The extracellular receptor kinase (ERK) pathway has been extensively characterized, is the most understood of the MAPK pathways (Denhardt 1996), and is a key pathway of carcinogenesis (Roberts and Der 2007).

In the present study, we extended our *in vitro* studies with F344 rat liver epithelial cells, which determined that PFOA, but not PFPeA, inhibited GJIC (Upham et al. 1998), to an *in vivo* study using F344 rats exposed to PFOA, PFPeA, or phenobarbital (PB), a known tumor promoter, to determine GJIC in liver tissue. We also continued our *in vitro* studies of PFOA versus PFPeA in determining differential effects of these compounds on MAPK, specifically ERK, and further determined that the mechanism of PFOA-induced inhibition of GJIC depends on redox activity, ERK, and phosphatidylcholine-specific phospholipase C (PC-PLC).

Materials and Methods

Chemicals. We purchased PFOA (purity > 90%) and PFPeA (purity = 97%), for the data presented in Figures 1–3 and 4A, from Fluka Chemie AG (Buchs, Switzerland), and because of unavailability from Fluka, we purchased PFOA for the data presented in Figures 4B, 5, and 6 from Aldrich Chemical Company Inc. (Milwaukee, WI, USA), with a purity of 96%. The purity values were obtained from the commercial sources. The ratios of linear versus branched isomers in our samples were undetermined. The stock solutions were prepared by dissolving the powder in the solvent: acetonitrile for the *in vitro* assays and dimethyl sulfoxide (DMSO) for the *in vivo* studies; we also used these solvents as the vehicle controls. We purchased Lucifer yellow (LY) from Molecular Probes (Eugene, OR, USA); sodium dodecyl sulfate, Tween 20, Tris, glycine, acrylamide, tetramethylethylenediamine (TEMED) and DC protein kit from Bio-Rad Laboratories (Hercules, CA, USA); DMSO, rhodamine-dextran (RhD; molecular weight, 10,000 Da), dithiothreitol (DTT), *N*-acetylcysteine (Nac), L-ascorbate-2-phosphate (Asc-2-P) sesquimagnesium salt hydrate, and PB from Sigma-Aldrich Chemical Company (St. Louis, MO, USA); D609 and U0126, from Tocris Bioscience (Ellisville, MO, USA); resveratrol from CTMedChem (Bronx, NY, USA); acetonitrile, from EM Science (Gibstown, NJ, USA); polyclonal antibodies directed to phospho-ERK, from New England Biolabs (Ipswich, MA, USA); and mouse polyclonal antibody directed to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), from Chemicon (Temecula, CA, USA).

In vivo *study*. Animal treatment. The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institutes of Health Sciences of Japan to assure that the rats were treated humanely and with regard for alleviation of suffering. Male Fischer-344 (F344) rats, 5 weeks old, were purchased from Charles River Japan (Kanagawa, Japan) and housed in plastic cages (five rats/cage). Male F-344 rats were chosen to match the *in vitro* studies that used liver epithelial cells isolated from male F-344 rats. The rats were kept under conditions of controlled temperature (23 ± 2°C), humidity (55 ± 5%), and lighting (12/12-hr dark/light cycle) and given CRF-12 basal diet (Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*.

We used the rats in the experiments after 1 week of acclimation. Eighty rats were divided into four groups and twenty rats per group were treated with a single intraperitoneal (i.p.) administration of 100 mg/kg PFOA, 100 mg/kg PFPeA, 100 mg/kg PB, or only vehicle (DMSO). Four rats per group were killed under anesthesia at 1, 3, 6, 12, and 24 hr after administration. Another 16 rats were divided into four groups and four rats of each group were given powder diet containing PFOA, PFPeA, PB, or basal powder diet only (control), and then killed after 1 week. The diets were prepared by blending each chemical into the basal powder diet at final concentrations of 0.02% for PFOA and PFPeA and 0.05% for PB. We determined the weight of the rats at the beginning and end of the experiment, and the food consumption on days 3 and 7 of the experiment. Based on the average weight of the rats and the average food consumed per day, the estimated daily doses of chemical exposures for PFOA, PFPeA, and PB were 37.9, 32.3, and 93.3 mg/day/kg, respectively.

Diethyl ether was used to euthanize the rats. Before sacrifice, blood was collected from the orbital venous plexus under anesthesia with diethyl ether and prepared for measuring serum aspartate aminotransferase (sAST), serum alanine aminotransferase (sALT), and serum alkaline phosphatase (sALP). Determination of sAST, sALT, and sALP was carried out with a Hitachi automatic Analyzer 7150 (Hitachi, Ltd., Tokyo, Japan) using commercially available GOP, GPT and ALP diagnostic reagents (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). After opening the abdominal cavity, we excised the liver and immediately used one part of the liver for the incision loading/dye transfer (IL/DT). Our preliminary study confirmed that the anesthetic and the vehicle, DMSO, under our experimental conditions did not affect *in vivo* GJIC.

Bioassay of GJIC (IL/DT). We assayed *ex vivo* GJIC in the liver by the IL/DT method described previously (Sai et al. 2000). A part of the left lobe of the liver was put on a plastic

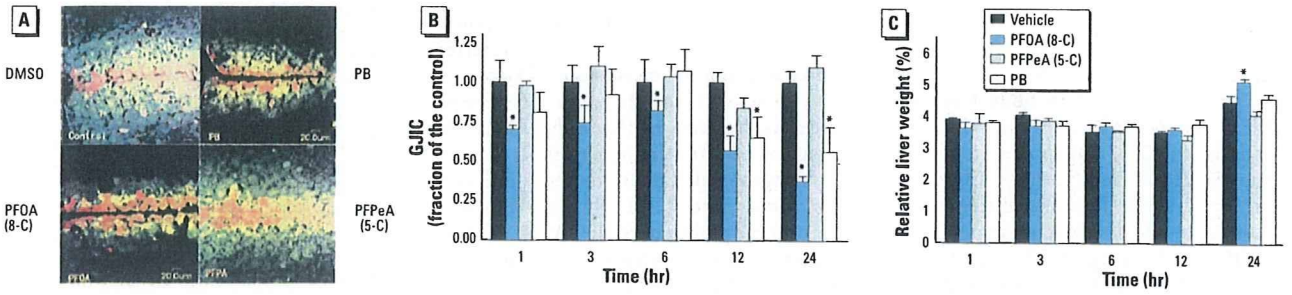


Figure 1. Analysis of *in vivo* effects of PFOA and PFPeA on GJIC in the liver tissue using IL/DT technique. Abbreviations: 5-C, five carbon; 8-C, eight carbon. (*A*) A fluorescent image of an IL/DT analysis of GJIC in the liver tissue of rats at 24 hr after a single i.p. administration of DMSO (vehicle), PB, PFOA, or PFPeA. Bar = 20 μm. (*B*) Mean + SD of the IL/DT data from rats treated with DMSO, PB, PFOA, or PFPeA for the acute exposure group. (*C*) Mean + SD relative liver weight from rats treated with DMSO, PB, PFOA, and PFPeA for the acute exposure group.
*$p < 0.05$ compared with vehicle, determined by one-way ANOVA for each time group followed by Dunnett's post hoc test.

plate covered with wet gauze. A mixture of fluorescent dyes containing 0.5 mg/mL LY and 0.5 mg/mL RhD in phosphate-buffered saline (PBS) was dropped on the tissue's surface. Three to four incisions were made on the surface of each specimen with a sharp blade. Excess amount of dye mixture was additionally put into the incisions and kept there for 3 min at room temperature. After incubation, the tissue was washed with PBS three times and fixed in 10% phosphate-buffered formalin overnight. Slices were washed with water and processed for embedding in paraffin. Five μm sections for GJIC analysis were prepared by cutting the paraffin block perpendicular to the incision line. Areas stained with LY alone or with RhD were detected by the emission of fluorescence using a confocal microscope (Fluoview, Olympus, Tokyo, Japan). We counted the number of cells stained with LY alone and normalized this number by dividing by the incision length. At least three incision sites per specimen were randomly chosen for the analysis, and the mean value was used as data from one animal. The values were expressed as a fraction of the control.

In vitro *study*. Cell culture. We obtained the WB-F344 rat liver epithelial cell line from J.W. Grisham and M.S. Tsao of the University of North Carolina at Chapel Hill, Chapel Hill, NC, USA (Tsao et al. 1984). Cells were cultured in D-medium (formula 78-5470EF, Gibco Laboratories, Grand Island, NY, USA), supplemented with 5% fetal bovine serum (Gibco Laboratories), and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The cells were grown in 35-mm tissue culture plates (Corning Inc., Corning, NY, USA) and the culture medium was changed every other day. Bioassays were conducted with confluent cultures that were obtained after 2–3 days of growth.

These WB cells are diploid and nontumorigenic (Tsao et al. 1984) and have been extensively characterized for GJIC in the absence and presence of well-known tumor promoters, growth factors, tumor suppressor genes, and oncogenes (Trosko and Ruch 1998). Intrahepatic transplantation of WB cells, which are liver bipolar stem cells, into adult syngenic F344 rats results in the morphologic differentiation of these cells into hepatocytes and incorporation into hepatic plates (Coleman et al. 1993).

Bioassay of GJIC (scrape load/dye transfer). The scrape loading/dye transfer (SL/DT) technique was adapted after the method of Upham et al. (1998). The test chemicals were added directly to the cell culture medium from concentrated stock solutions. The migration of the dye through gap junctions was visualized with a Nikon Eclipse TE3000 phase contrast/fluorescent microscope and the images were digitally captured with Nikon EZ Cool Snap charge-coupled device camera (Nikon Inc., Nikon, Japan). GJIC was assessed by comparing the distance the dye traveled in the chemically treated cells with the distance the dye traveled in the vehicle controls, which was measured using the Gel-Expert imaging software (Nucleotech, San Mateo, CA, USA). We report GJIC as a fraction of the control. Based on previous results (Upham et al. 1996, 1998), 1-methylanthracene as well as PFOA were used as positive controls of inhibition of GJIC, whereas acetonitrile at vehicle concentrations was used as a negative control. The vehicles used for the *in vitro* assays, acetonitrile and PBS, had no effect on GJIC. We performed all experiments at least in triplicate and report the results as means ± SD at the 95% confidence interval.

Western blot analysis. Cells were grown in 35-mm-diameter Corning tissue culture plates to the same confluency as the SL/DT assay. The cells were depleted of serum 5 hr before addition of PFFAs to synchronize the cells into G_0 to minimize background ERK levels. This does not alter the effect on GJIC in the F344 WB cells, as previously determined (Rummel et al. 1999). The proteins were extracted with 20% sodium dodecyl sulfate (SDS) solution containing 1 mM phenylmethylsulfonyl fluoride, 100 μM Na_3VO_4, 100 nM aprotinin, 1.0 μM leupeptin, 1.0 μM antipain, and 5.0 mM NaF. The protein content was determined with the Bio-Rad DC assay kit. The proteins were separated on 12.5% SDS–polyacrylamide gel electrophoresis according to the method of Laemmli

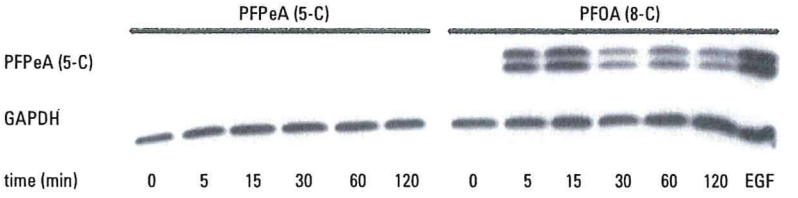


Figure 3. Activation of ERK-MAPK by PFOA, but not by PFPeA, in F344 WB rat liver epithelial cells determined by Western blots: Top panel probed with a phosphorylated ERK specific antibody and the bottom panel probed with a GAPDH specific antibody. The concentrations of PFPeA and PFOA were 100 μM. The concentration and time of incubation for epidermal growth factor (EGF) was 20.0 ng/mL and 15 min.

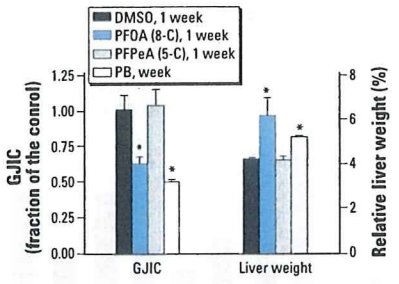


Figure 2. The long-term effects (1 week) of PB, PFOA, and PFPeA on GJIC and RLW (mean + SD). Abbreviations: 5-C, five carbon; 8-C, eight carbon. A one-way ANOVA was done for the GJIC data and a Kruskal-Wallis one-way ANOVA was done for the RLW data because these data failed the normality test.

*$p < 0.05$ compared with vehicle (DMSO); significant effects determined by ANOVA or Kruskal-Wallis ANOVA for each group was followed with a Dunnett's post hoc test at $p < 0.05$.

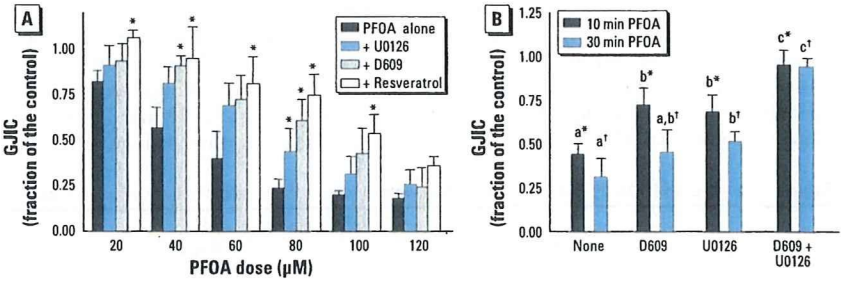


Figure 4. (*A*) Prevention of PFOA-induced inhibition of GJIC by inhibitors of MEK and PC-PLC and resveratrol at various doses of PFOA (mean + SD). The concentrations and times of preincubation of U0126, D609, and resveratrol were 20 μM/30 min, 50 μM/20 min, and 100 μM/15 min, respectively. A one-way ANOVA was done for each dose group. *Significant at $p < 0.05$ using the Dunnett's post hoc test that compared each inhibitor treatment with that of PFOA alone. (*B*) The interactive effect of MEK and PC-PLC inhibitors on reversing PFOA-induced inhibition of GJIC at 10 and 30 min (mean + SD). The concentrations and times of preincubation of U0126 and D609 were 20 μM/30 min and 50 μM/20 min, respectively. A one-way ANOVA indicated significance at $p < 0.05$ for each time group. The Tukey pairwise-comparison post hoc test was used to determine statistical differences, as indicated by different letters, between the inhibitor treatments for each time group. The lettered asterisks represent the 10-min group and lettered daggers represent the 30-min group.

(1970). Fifteen micrograms protein was loaded onto the gels and electrophoretically transferred from the gel to polyvinyl difluoride membranes (Millipore Corp., Bedford, MA, USA). Phosphorylated ERK 1 and ERK 2 were detected with a 1:2,000 dilution of anti-phospho-ERK polyclonal antibodies, and GAPDH was detected with a 1:10,000 dilution of anti-GAPDH polyclonal antibodies, that were incubated sequentially with the membranes, each for 2 hr. The protein–primary antibody complex was probed with a 1:1,000 dilution of horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibodies (Amersham Life Science Products, Arlington Heights, IL, USA) for 1 hr. The ERK and GAPDH protein bands were detected using the Super Signal chemiluminescence detection kit (Pierce Corp., Arlington Heights, IL, USA), enhanced chemiluminescence (ECL) detection kit, and ECL Hyperfilm–MP (Amersham Life Science Products, Denver, CO, USA).

Statistics. For the *in vivo* studies, the value of each group was expressed as the mean ± SD of data derived from four rats. The *in vitro* assays were done in at least triplicate and expressed as a fraction of the control. The significance of differences in all results was evaluated with either a one-way analysis of variance (ANOVA) or, if the data set failed the normality test, a Kruskal-Wallis one-way ANOVA on ranked means. Normality assumption testing was done with the Kolmogorov-Smirnov test and equal variance assumption testing with the Levene median test. If ANOVA or Kruskal-Wallis ANOVA rejected the null hypothesis, then the results that were compared with a designated control used Dunnett's multiple-comparison post hoc tests or Tukey's post hoc test for pairwise multiple comparisons.

Results

In vivo *results.* The *in vivo* results of PFOA and PFPeA were compared with PB, a known liver tumor promoter. We used two different dosing schemes: an acute 24-hr exposure via i.p. administration and a longer-term (1 week) dietary exposure. An ANOVA indicated that PFOA, PFPeA, and PB had no statistically significant effect on body weights of the rats (data not shown). Liver injury was assessed using the biomarkers sALT, sAST, sALP, and the results for both dosing schemes are presented in Table 1. At day 7, there were no significant differences between the rats treated with PFOA, PFPeA, and PB for all three of the selected liver enzymes, indicating no long-term liver injury. After 1 day, we found a small, biologically insignificant, but statistically significant increase in sAST, with the data exhibiting high variability.

To assess the *in vivo* effects of these compounds on GJIC in the liver tissue, we used an IL/DT technique. Figure 1A shows the incorporation of the fluorescent dye into the liver cells and subsequent distribution of the fluorescent dye through the gap junctions of the tissue. RhD, which is a large-molecular-weight dye that does not traverse gap junctions, is color-coded red. LY, which does travel through gap junction channels, is color-coded from yellow for high intensity to green for lower intensity. We measured and averaged the distances traveled by the gap-junction–permeable dye and show them in Figure 1B (acute exposure) and Figure 2 (long-term exposure). PFOA and PB but not PFPeA inhibited *in vivo* GJIC in the liver tissues of rats treated either acutely or chronically. Significant inhibition of GJIC by PFOA was observed after 1 hr, and continued to inhibit GJIC until 24 hr in the acutely treated rats. Significant inhibition of GJIC did not begin until after 12 hr of treatment with PB in this group of rats.

In the acute dose regimen (Figure 1C), a significant increase in the relative weight of livers from rats treated with PFOA was observed at 24 hr. Similarly, rats chronically exposed to PFOA and PB for 1 week had significant increases in relative liver weight (RLW; Figure 2). The livers of animals treated either acutely or chronically with PFPeA did not significantly increase in relative weights compared with rats fed the vehicle (Figures 1C, 2).

In vitro *results.* Considering that the *in vitro* results of PFOA and PFPeA effects on gap junctions correlated with their effects on gap junctions *in vivo*, we did further *in vitro* analyses of PFOA to determine underlying mechanisms involved in the dysregulation of GJIC. PFOA, which inhibits GJIC, also activated ERK as determined by Western blot analysis of the phosphorylated, activated form of ERK (Figure 3). In contrast, the non-GJIC inhibitory PFPeA did not activate ERK (Figure 3). Activation of ERK was within 5 min in cells treated with PFOA, which correlates with the time of inhibition of GJIC, indicating a potential link. Preincubation of the cells with an MEK inhibitor, U0126, partially but significantly prevented the inhibition of GJIC by PFOA (Figure 4A). Preincubation of the cells with the PC-PLC inhibitor D609 also partially but significantly prevented the inhibition of GJIC by PFOA (Figure 4A). The significant contribution of PC-PLC and MEK in PFOA-induced inhibition of GJIC diminished after the maximum inhibitory dose of 80 μM to a nonsignificant involvement at the higher dose of 120 μM (Figure 4A), indicating further that mechanisms other than MEK and PC-PLC are also involved.

Gap junctions are known to be redox sensitive, so we conducted several experiments with various antioxidants. Resveratrol significantly reversed the inhibitory effect on GJIC and was possibly inhibiting both MEK and PC-PLC (Figure 4A). Additional experiments were performed to look at the combinatorial effect of pretreating cells with both D609 and U0126. The combination of both of these inhibitors of signal transduction enzymes resulted in the prevention of GJIC inhibition by PFOA, and the combinatorial effect was significantly greater than cells treated with either inhibitor alone as determined by a Tukey post hoc multiple-comparison test (Figure 4B). These results collectively indicate that PFOA-induced regulation of GJIC is a function of both of these signaling enzymes.

Further experiments were performed with DTT, Nac, and Asc-2-P (Figure 5). DTT

Table 1. The effect of PFOA, PFPeA, and PB on the levels of various biomarkers of liver injury in F344 rats.

Exposure, time, enzyme	Enzyme activity (mU/mL) DMSO (vehicle)	PFOA	PFPeA	PB
Acute (24 hr)				
sALT	51.5 ± 3.2	138.6 ± 126.4	56.3 ± 13.2	54.5 ± 4.1
sAST	98.8 ± 8.8	232 ± 169.8*	113.0 ± 17.6	100.6 ± 15.1
sALP	1672.8 ± 90.0	1521.8 ± 220.2	1495.0 ± 233.8	1561.0 ± 115.2
Longer-term (1 week)				
sALT	39.3 ± 2.0	41.2 ± 1.9	39.8 ± 3.0	39.7 ± 2.6
sAST	71.2 ± 10.0	70.4 ± 4.3	73.9 ± 10.7	76.1 ± 9.4
sALP	1488.8 ± 62.9	1394.5 ± 59.4	1449.5 ± 36.6	1349.3 ± 53.0

To determine significant effects, we performed a one-way ANOVA for sALP (1 day), sALP (1 week), and sALT (1 week) and a Kruskal-Wallis one-way ANOVA on ranks for sAST (1 day), sAST (1 week), and sALT (1 day). Any significant effects determined by ANOVA were followed by a Dunnett's post hoc test, with DMSO designated as the control.
*$p < 0.05$.

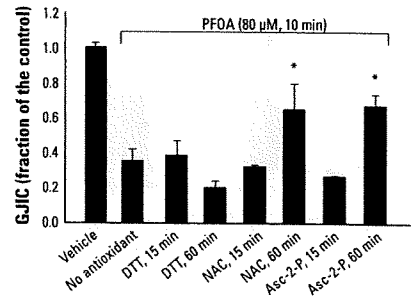


Figure 5. Prevention of PFOA-induced inhibition of GJIC by various antioxidants (mean + SD). The concentrations of PFOA, DTT, Nac, and Asc-2-P were 80 μM, 10 mM, 100 μM, and 100 μM, respectively.
*$p < 0.05$ by ANOVA and Dunnett's post hoc test comparing each antioxidant treatment with that of PFOA alone (no antioxidant).

and Nac in the absence of PFOA had no statistically (ANOVA) significant effect on GJIC at both 15 and 60 min (data not shown). Asc-2-P had a small, < 10% effect (ANOVA, Tukey) on GJIC in the absence of PFOA at 15 min but not 60 min (data not shown). Asc-2-P and Nac both prevented the inhibition of GJIC by PFOA within a 60-min preincubation time, but not DTT, implicating redox-sensitive proteins that probably do not involve thiol oxidations. Preincubation of Asc-2-P and Nac for 15 min did not reverse the effect of PFOA on GJIC. The oxidative nature of PFOA was not cytotoxic, as indicated after 2 days of growing cells after the log-phase of growth with 80 μM PFOA, resulting in no visual abnormalities in the morphology of the cells and complete restoration of GJIC after the cells were transferred to fresh medium for 5 hr containing no PFOA (Figure 6).

Discussion

Understanding the biological effects of the environmentally prevalent PFFAs on cell signaling pathways relevant to the epigenetic, nongenotoxic phase of cancer is important. In particular, GJIC offers a very central signaling system to assess risk (Trosko and Upham 2005). Although the transient closure of gap junction channels during proliferation is a normal response to mitogens, the chronic inhibition of GJIC by toxicants and toxins or by cytokines released during compensatory hyperplasia could lead to pathologic states (Trosko and Upham 2005; Upham and Trosko 2006). Thus, we conducted two dosing schemes, one a short term of 24 hr following an i.p. injection of PFOA, PFPeA, or PB, and another a longer-term study where the rats were dosed with these compounds through their daily feedings for 1 week. We previously demonstrated that inhibition of GJIC using *in vitro* model systems by perfluoroalkyl carboxylates and sulfonates depended on the chain length, where PFFAs with 7–10 carbons inhibited GJIC, and PFFAs with 2–6 carbons did not (Hu et al. 2002; Upham et al. 1998). To determine if chain length of PFFAs would exhibit similar effects on GJIC in a living organism, we treated F344 rats with PFOA, an eight-carbon PFFA, and PFPeA, a five-carbon PFFA, and determined GJIC in the liver tissue using an *ex vivo* IL/DT assay.

The liver is the primary target of PFOA (Kudo and Kawashima 2003), which is known to induce hepatocellular tumors in rodent model systems (Abdellatif et al. 1991; Kennedy et al. 2004). Similar to our *in vitro* results (Hu et al. 2002; Upham et al. 1998), PFOA decreased GJIC activity in the liver compared with the rats treated with the vehicle (control) for both the acute and long-term dosing schemes. In contrast, PFPeA-treated rats did not have altered GJIC in the livers compared with the control rats for both dosing schemes, which is also consistent with our *in vitro* observations. Another possible reason for the lack of an *in vivo* response by PFPeA could be a consequence of a greater elimination rate that is typical of PFFAs with shorter chain lengths (Chang et al. 2008; Ohmori et al. 2003). Although we did not measure the elimination rates of PFPeA in our experiments, the half-life of perfluorobutyrate is 9.2 hr (oral) and 6.4 hr (intravenous) in Sprague-Dawley rats (Chang et al. 2008). These half-lives are similar to that of PB in Sprague-Dawley rats, which is 8–9 hr. Considering that PB inhibited GJIC and induced hepatomegaly in the livers of the rats used in our experiments, and PFPeA did not inhibit GJIC using an *in vitro* assay system, we would expect that the noninhibitory effects of PFPeA on GJIC *in vivo* would not result from its increased rate of elimination. Further experiments are needed to confirm such a conclusion.

We previously published data that indicated the treatment of Sprague-Dawley rats with PFOS resulted in a decrease in GJIC activity in the liver tissue; thus, PFOA and PFOS have similar activities (Hu et al. 2002). The following are additional reports demonstrating that tumor promoters, known to inhibit GJIC *in vitro*, also inhibited GJIC *in vivo*: pentachlorophenol (Sai et al. 2000), 2-acetylaminofluorene (Krutovskikh et al. 1991), PB (Kolaja et al. 2000; Krutovskikh 1995), polychlorinated biphenyls (Kolaja et al. 2000; Krutovskikh 1995), pregnenolone-16α-carbonitrile (Kolaja et al. 2000), cadmium (Jeong et al. 2000), clofibrate, and DDT (Krutovskikh 1995). Another interesting report on the *in vivo* effects of chemicals on GJIC is the treatment of rats with the antioxidants lycopene and alpha and beta carotene. High doses of these antioxidants resulted in a decrease in GJIC activity, whereas rats exposed to low doses exhibited an increase in GJIC (Krutovskikh et al. 1997). Although *in vivo* assessment of intercellular communication has been limited in both the number of studies and choice of organ, namely, the liver, these results, including those presented in this report, nevertheless suggest that the *in vitro* rat liver epithelial cell assay system is a good predictor of the *in vivo* effects of chemicals on gap junctions in the liver tissues of rodents.

PFOA and PB induced hepatomegaly, whereas PFPeA had no effect. These results are similar to those previously published indicating that PFOA, but not perfluorobutyrate, affected RLWs in F344 rats (Takagi et al. 1991). Although not causally linked, hepatomegaly has been correlated with the promotion of liver tumors by many peroxisome proliferator-activated receptor α agonists, including PFOA (Takagi et al. 1992). The null effect of PFPeA on GJIC and hepatomegaly suggest that PFPeA would not be a tumor promoter; however, two-stage (initiation and promotion) carcinogenesis studies would be needed to confirm this conclusion. Tissue necrosis is known to induce compensatory hyperplasia that leads to increased liver weights, but this is unlikely the cause of hepatomegaly in the PFOA- and PB-treated rats, considering that no visual damage of the liver was seen in the histologic sections (data not shown) and there was no increase in serum enzymes.

Tissue homeostasis in multicellular organisms depends on functional GJIC, and the disruption of intercellular communication has been linked to many diseases (Trosko and Upham 2005). PFOA clearly interrupted GJIC in the liver tissues of rats, but further experiments would need to be done in other species. PFOS also inhibited GJIC in rat liver tissue as well as *in vitro* systems that included dolphin kidney cells (Hu et al. 2002). Thus, the potential for cross-species effects of PFOA on GJIC implicates a health risk to multicellular organisms. Future experiments, particularly with human cell lines, will aid in determining differences in the sensitivity of various organisms to the effects of PFOS and PFOA on GJIC and allow for more accurate assessment of risks these compounds pose to humans and wildlife.

Considering that *in vitro* analyses of PFFA, using rat liver epithelial cells, accurately predicted the *in vivo* effects on GJIC for various PFFAs, we did further *in vitro* analyses of PFPeA- and PFOA-treated rat liver epithelial cells to determine potential signaling mechanisms involved in PFOA-induced regulation of GJIC. Connexin 43 (Cx43) is a

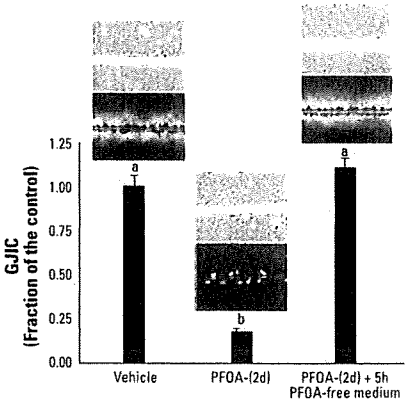


Figure 6. The effects of an extended incubation of cells with PFOA (80 μM, 2 days) and transfer of cells to PFOA-free medium (5 hr) on cell morphology and GJIC (mean + SD). Each phase-contrast and fluorescent photomicrograph represents one of the three replicates of each treatment group (magnification, 200×). Different letters indicate significance at $p < 0.05$ using ANOVA and Tukey post hoc test with a pairwise comparison.

phosphoprotein, and the phosphorylation of the carboxy terminus by protein kinases, such as protein kinase C (PKC), Src, and MAPKs, in the regulation of GJIC has been well documented (Solan and Lampe 2005). Although phosphorylation of gap junctions is known to regulate the function, assembly, internalization, and degradation of this protein complex, the alteration of connexin phosphorylation by protein kinases, such as MAPKs, does not necessarily dysregulate gap junction function (Hossain et al. 1999), nor does the activation of protein kinases (i.e., MAPK) alter the phosphorylation status of connexins (Upham et al. 2008).

This was also true for PFOA, which clearly activated ERK-MAPK (Figure 3) but did not induce a change in the phosphorylation pattern of Cx43 as previously determined by Western blot analysis (Upham et al. 1998). Whether or not gap junctions are phosphorylated, several compounds (i.e., growth factors, lindane, lysophosphatidic acid, 12-*O*-tetradecanoylphorbol-13-acetate, and cannabinoids) are known to inhibit GJIC through a MEK-dependent pathway (Komatsu et al. 2006; Mograbi et al. 2003; Rivedal and Opsahl 2001; Upham et al. 2003). Although many compounds activate MAPKs, such as p38 and ERK, the mechanism of inhibiting GJIC by many of these compounds is independent from these MAPKs (Machala et al. 2003; Upham et al. 2008).

Our results indicated that PFOA activated ERK in F344 WB rat liver epithelial cells within 5 min, and this time period is within the interval required for the inhibition of GJIC by PFOA in this cell line. PFPeA, which does not inhibit GJIC in this cell line (Upham et al. 1998), also did not activate ERK. Preincubation of these cells with an MEK inhibitor, U0126, partially prevented PFOA from inhibiting GJIC, indicating that PFOA-induced modulation of GJIC was not solely dependent on the ERK pathway.

Recently, PC-PLC has been implicated in the dysregulation of GJIC in response to toxicants that regulate GJIC through an MEK-independent mechanism (Machala et al. 2003; Upham et al. 2008). Preincubation of F344 WB cells with the PC-PLC inhibitor D609 also partially prevented PFOA from inhibiting GJIC. These results suggest that PFOA is regulating GJIC through multiple cellular mechanisms. This becomes more apparent as the dose of PFOA is increased resulting in the inhibition of GJIC at a high dose of 120 μM that depended on neither PC-PLC nor MEK. However, maximum inhibition of GJIC by PFOA, which was around 80 μM, was very dependent on the activity of both MEK and PC-PLC. This was further apparent from the experiment where cells were pretreated with a combination of both D609 and U0126, resulting in almost complete recovery of GJIC. The activation of ERK and PC-PLC will not only control gap junction function but is known to alter gene expression, leading to various pathologies, including cancer. The function of PC-PLC in tumorigenesis has not been extensively studied, yet there are significant reports indicating that PC-PLC does play a very significant role in cancer (Cheng et al. 1997). The ERK pathway has been extensively characterized and is the most understood of the MAPK pathways (Denhardt 1996) and is a key pathway of carcinogenesis (Roberts and Der 2007).

PFOA, but not perfluorobutyrate, is known to induce oxidative stress in the livers of rats, as indicated by 8-hydroxydeoxyguanosine formation (Takagi et al. 1991), and redox mechanisms are known to commonly play a role in gap junction function (Upham and Trosko 2009). These oxidative signaling effects could be site-directed redox regulations of specific regulatory proteins or from general oxidative effects (Upham and Trosko 2008). Recently, we reported that the antioxidant resveratrol prevented inhibition of GJIC by dicumylperoxide but not by benzoylperoxide (Upham et al. 2007). Dicumylperoxide, but not benzoylperoxide, inhibits GJIC through a PC-PLC–dependent mechanism (Upham et al. 2007). Similar to dicumylperoxide, we showed that resveratrol prevented inhibition of GJIC by PFOA to a greater level than either D609 or U0126 alone, but similar to the level of GJIC recovery seen when cells were pretreated with both D609 and U0126. These results indicate the possibility that PFOA dysregulates GJIC through both MEK and PC-PLC and that protection of GJIC by resveratrol is potentially through oxidative signaling events controlling both MEK and PC-PLC. Beyond the implication of redox mechanisms of the resveratrol experiment, this antioxidant is regularly consumed by humans and is found in high concentrations in red wine and peanut products (Sobolev and Cole 1999; Wang et al. 2002), and thus may have some relevance to the health of humans that may be exposed to environmental toxicants, such as PFOA. Chemopreventive effects of resveratrol are known to inhibit initiation, promotion, and progression of tumors (Signorelli and Ghidoni 2005). Thus, resveratrol could potentially contribute to a protective effect in humans exposed to PFOA by significantly blocking PFOA from inhibiting GJIC.

The addition of Asc-2-P or Nac partially reversed the inhibitory effects of PFOA on GJIC, similar to that of resveratrol. In contrast, DTT did not prevent PFOA from inhibiting GJIC, indicating that the oxidative events controlling PC-PLC and Mek are not thiol based. The exposure of F344 WB cells to PFOA for 2 days showed no adverse effects on cell morphology, and they communicated normally after PFOA was removed from the medium (Figure 6), which implicates that the PFOA-induced oxidative events are not killing the cells. These results suggest that general oxidative processes are involved in PFOA-induced inhibition of GJIC and that health benefits could potentially be attained by the consumption of many antioxidant rich foods, particularly in individuals deficient in antioxidants. Moreover, the reversible properties of PFOA-induced inhibition of GJIC are consistent with the known reversible nature of tumor promoters in two-stage carcinogenesis model systems (Trosko and Upham 2005). These results also indicate that reversing the effect of PFOA on GJIC after a simple washing of the treated cells with PBS demonstrates that PFOA is not covalently or tightly bound to the cell. The effect of PFOA on GJIC was probably not a consequence of directly interacting with the gap junction proteins because the inhibition of MAPK and PC-PLC both prevented the GJIC effect. Possibly PFOA interacted with these two proteins or interacted with a signaling protein or receptor even further upstream.

In conclusion, the *in vitro* assay system used to assess the effects of PFOA and PFPeA on GJIC predicted the *in vivo* results of GJIC from rats treated with these compounds. GJIC plays a vital role in maintaining tissue homeostasis, and disruption of gap junction function can lead to diseased states such as tumorigenesis. These results are similar to other tumor-promoting compounds tested in both an *in vitro* and *in vivo* assay system. Although there are several mechanisms by which environmental compounds might promote an initiated cell, such as through peroxisome proliferator activated receptors or protein kinase C, the disruption of normal intercellular communication is an essential event of multiple tumorigenic mechanisms (Trosko and Upham 2005) and serves as a central biomarker to assess the epigenetic toxicity of contaminants (Rosenkranz et al. 1997; Trosko and Upham 2005), as well as to assess the potential antitumorigenic health benefits of nutrition based food products (Trosko and Upham 2005).

References

Abdellatif AG, Preat V, Taper HS, Roberfroid M. 1991. The modulation of rat liver carcinogenesis by perfluorooctanoic acid, a peroxisome proliferator. Toxicol Appl Pharmacol 111(3):530–537.

Borek C, Sachs L. 1966. The difference in contact inhibition of cell replication between normal cells and cells transformed by different carcinogens. Proc Natl Acad Sci USA 56(1705):1705–1711.

Calafat AM, Wong LY, Kuklenyik Z, Reidy JA, Needham LL. 2007. Polyfluoroalkyl chemicals in the U.S. population: data from the National Health and Nutrition Examination Survey (NHANES) 2003–2004 and comparisons with NHANES 1999–2000. Environ Health Perspect 115:1596–1602.

Cattley RC, Miller RT, Corton JC. 1995. Peroxisome proliferators: potential role of altered hepatocyte growth and

differentiation in tumor development. Prog Clin Biol Res 391:295–303.

Chang SC, Das K, Ehresman DJ, Ellefson ME, Gorman GS, Hart JA, et al. 2008. Comparative pharmacokinetics of perfluorobutyrate in rats, mice, monkeys, and humans and relevance to human exposure via drinking water. Toxicol Sci 104(1):40–53.

Cheng J, Weber JD, Baldassare JJ, Raben DM. 1997. Ablation of Go alpha-subunit results in a transformed phenotype and constitutively active phosphatidylcholine-specific phospholipase C. J Biol Chem 272(28):17312–17319.

Coleman WB, Wennerberg AE, Smith GJ, Grisham JW. 1993. Regulation of the differentiation of diploid and some aneuploid rat liver epithelial (stemlike) cells by the hepatic microenvironment. Am J Pathol 142(5):1373–1382.

Denhardt DT. 1996. Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. Biochem J 318(pt 3):729–747.

DeWitt JC, Copeland CB, Strynar MJ, Luebke RW. 2008. Perfluorooctanoic acid–induced immunomodulation in adult C57BL/6J or C57BL/6N female mice. Environ Health Perspect 116:645–650.

Hekster FM. 2003. Environmental and toxicity effects of perfluoroalkylated substances. Rev Environ Contam Toxicol 79:99–121.

Hossain MZ, Jagdale AB, Ao P, Boynton AL. 1999. Mitogen-activated protein kinase and phosphorylation of connexin43 are not sufficient for the disruption of gap junctional communication by platelet-derived growth factor and tetradecanoylphorbol acetate. J Cell Physiol 179(1):87–96.

Hu W, Jones PD, Upham BL, Trosko JE, Lau C, Giesy JP. 2002. Inhibition of gap junctional intercellular communication by perfluorinated compounds in rat liver and dolphin kidney epithelial cell lines in vitro and Sprague-Dawley rats in vivo. Toxicol Sci 68(2):429–436.

Jeong SH, Habeebu SSM, Klaassen CD. 2000. Cadmium decreases gap junctional intercellular communication in mouse liver. Toxicol Sci 57(1):156–166.

Kannan K. 2001. Accumulation of perfluorooctane sulfonate in marine mammals. Environ Sci Technol 35(8):1593–1598.

Kennedy GL, Butenhoff JL, Olsen GW, O'Connor JC, Seacat AM, Perkins RG, et al. 2004. The toxicology of perfluorooctanoate. Crit Rev Toxicol 34(4):351–384.

Key BD, Howell RD, Criddle CS. 1997. Fluorinated organics in the biosphere. Environ Sci Technol 31:2445–2454.

Klaunig JE, Babich MA, Baetcke KP, Cook JC, Corton JC, David RM, et al. 2003. PPARalpha agonist-induced rodent tumors: modes of action and human relevance. Crit Rev Toxicol 33(6):655–780.

Kolaja KL, Engelken DT, Klaassen CD. 2000. Inhibition of gap-junctional-intercellular communication in intact rat liver by nongenotoxic hepatocarcinogens. Toxicology 146(1):15–22.

Komatsu J, Yamano S, Kuwahara A, Tokumura A, Irahara M. 2006. The signaling pathways linking to lysophosphatidic acid-promoted meiotic maturation in mice. Life Sci 79(5):506–511.

Krutovskikh VA. 1995. Inhibition of rat liver gap junction intercellular communication by tumor-promoting agents in vivo. Association with aberrant localization of connexin proteins. Lab Invest 72(5):571–577.

Krutovskikh V, Asamoto M, Takasuka N, Murakoshi M, Nishino H, Tsuda H. 1997. Differential dose-dependent effects of alpha-, beta-carotenes and lycopene on gap-junctional intercellular communication in rat liver in vivo. Jpn J Cancer Res 88(12):1121–1124.

Krutovskikh VA, Oyamada M, Yamasaki H. 1991. Sequential changes of gap-junctional intercellular communications during multistage rat liver carcinogenesis: direct measurement of communication in vivo. Carcinogenesis 12(9):1701–1706.

Kudo N, Kawashima Y. 2003. Toxicity and toxicokinetics of perfluorooctanoic acid in humans and animals. J Toxicol Sci 28(2):49–57.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Machala M, Blaha L, Vondracek J, Trosko JE, Scott J, Upham BL. 2003. Inhibition of gap junctional intercellular communication by noncoplanar polychlorinated biphenyls: inhibitory potencies and screening for potential mode(s) of action. Toxicol Sci 76(1):102–111.

Mograbi B, Corcelle E, Defamie N, Samson M, Nebout M, Segretain D, et al. 2003. Aberrant connexin 43 endocytosis by the carcinogen lindane involves activation of the ERK/mitogen-activated protein kinase pathway. Carcinogenesis 24(8):1415–1423.

Ohmori K, Kudo N, Katayama K, Kawashima Y. 2003. Comparison of the toxicokinetics between perfluorocarboxylic acids with different carbon chain length. Toxicology 184(2–3):135–140.

Potter VR. 1978. Phenotypic diversity in experimental hepatomas: the concept of partially blocked ontogeny. Br J Cancer 38(1):1–23.

Rivedal E, Opsahl H. 2001. Role of PKC and MAP kinase in EGF- and TPA-induced connexin43 phosphorylation and inhibition of gap junction intercellular communication in rat liver epithelial cells. Carcinogenesis 22(9):1543–1550.

Roberts PJ, Der CJ. 2007. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 26(22):3291–3310.

Rosenkranz M, Rosenkranz HS, Klopman G. 1997. Intercellular communication, tumor promotion and non-genotoxic carcinogenesis: relationships based upon structural considerations. Mutat Res 381(2):171–188.

Rummel AM, Trosko JE, Wilson MR, Upham BL. 1999. Polycyclic aromatic hydrocarbons with bay-like regions inhibited gap junctional intercellular communication and stimulated MAPK activity. Toxicol Sci 49(2):232–240.

Sai K, Kanno J, Hasegawa R, Trosko JE, Inoue T. 2000. Prevention of the down-regulation of gap junctional intercellular communication by green tea in the liver of mice fed pentachlorophenol. Carcinogenesis 21(9):1671–1676.

Signorelli P, Ghidoni R. 2005. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. J Nutr Biochem 16(8):449–466.

Skutlarek D, Exner M, Farber H. 2006. Perfluorinated surfactants in surface and drinking waters. Environ Sci Pollut Res Int 13(5):299–307.

Sobolev VS, Cole RJ. 1999. trans-resveratrol content in commercial peanuts and peanut products. J Agric Food Chem 47(4):1435–1439.

Solan JL, Lampe PD. 2005. Connexin phosphorylation as a regulatory event linked to gap junction channel assembly. Biochim Biophys Acta 1711(2):154–163.

Takagi A, Sai K, Umemura T, Hasegawa R, Kurokawa Y. 1991. Short-term exposure to the peroxisome proliferators, perfluorooctanoic acid and perfluorodecanoic acid, causes significant increase of 8-hydroxydeoxyguanosine in liver DNA of rats. Cancer Lett 57(1):55–60.

Takagi A, Sai K, Umemura T, Hasegawa R, Kurokawa Y. 1992. Hepatomegaly is an early biomarker for hepatocarcinogenesis induced by peroxisome proliferators. J Environ Pathol Toxicol Oncol 11:145–149.

Tao L. 2006. Perfluorooctanesulfonate and related fluorochemicals in albatrosses, elephant seals, penguins, and polar skuas from the Southern Ocean. Environ Sci Technol 40(24):7642–7648.

Trosko JE, Ruch RJ. 1998. Cell-cell communication in carcinogenesis. Front Biosci 3:208–236.

Trosko JE, Ruch RJ. 2002. Gap junctions as targets for cancer chemoprevention and chemotherapy. Curr Drug Targets 3(6):465–482.

Trosko JE, Upham BL. 2005. The emperor wears no clothes in the field of carcinogen risk assessment: ignored concepts in cancer risk assessment. Mutagenesis 20(2):81–92.

Tsao MS, Smith JD, Nelson KG, Grisham JW. 1984. A diploid epithelial cell line from normal adult rat liver with phenotypic properties of "oval" cells. Exp Cell Res 154(1):38–52.

Upham BL, Blaha L, Babica P, Park JS, Sovadinova I, Pudrith C, et al. 2008. Tumor promoting properties of a cigarette smoke prevalent polycyclic aromatic hydrocarbon as indicated by the inhibition of gap junctional intercellular communication via phosphatidylcholine-specific phospholipase C. Cancer Sci 99(4):696–705.

Upham BL, Deocampo ND, Wurl B, Trosko JE. 1998. Inhibition of gap junctional intercellular communication by perfluorinated fatty acids is dependent on the chain length of the fluorinated tail. Int J Cancer 78(4):491–495.

Upham BL, Guzvic M, Scott J, Carbone JM, Blaha L, Coe C, et al. 2007. Inhibition of gap junctional intercellular communication and activation of mitogen-activated protein kinase by tumor-promoting organic peroxides and protection by resveratrol. Nutr Cancer 57(1):38–47.

Upham BL, Rummel AM, Carbone JM, Trosko JE, Ouyang Y, Crawford RB, et al. 2003. Cannabinoids inhibit gap junctional intercellular communication and activate ERK in a rat liver epithelial cell line. Int J Cancer 104(1):12–18.

Upham BL, Trosko JE. 2006. A paradigm shift in the understanding of oxidative stress and it's implications to exposure of low-level ionizing radiation. Acta Med Nagasaki 50:63–68.

Upham BL, Trosko JE. 2009. Oxidative-dependent integration of signal transduction with intercellular gap junctional communication in the control of gene expression. Antioxid Redox Signal 11(2):297–307.

Upham BL, Weis LM, Rummel AM, Masten SA, Trosko JE. 1996. The effects of anthracene and methylated anthracenes on gap junctional intercellular communication in rat liver epithelial cells. Fundam Appl Toxicol 34(2):260–264.

Van de Vijver KI. 2005. Tissue distribution of perfluorinated chemicals in harbor seals (*Phoca vitulina*) from the Dutch Wadden Sea. Environ Sci Technol 39(18):6978–6984.

Wang Y, Catana F, Yang Y, Roderick R, van Breemen RB. 2002. An LC-MS method for analyzing total resveratrol in grape juice, cranberry juice, and in wine. J Agric Food Chem 50(3):431–435.