The role of endogenous aryl hydrocarbon receptor signaling in cardiovascular physiology

Nan Zhang

*Department of Internal Medicine, Division of Cardiovascular Medicine, University of Michigan
Medical School, USA*
Address for correspondence: *Dr. Nan Zhang, Department of Internal Medicine, 1150,
West Medical Center Drive, Room 7301, Ann Arbor, MI 48109-0644, USA. E-mail: nanz@umich.edu*

ABSTRACT

The aryl hydrocarbon receptor (AHR) is an orphan nuclear receptor with a primary function of mediating xenobiotic metabolism through transcriptional activation of Phase I and Phase II drug-metabolizing enzymes. Although no high-affinity physiological activators of AHR have been discovered, the endogenous signaling of the AHR pathway is believed to play an important role in the development and function of the cardiovascular system, based on the observations on *ahr* gene-deficient mice. The AHR knockout mice develop cardiac hypertrophy, abnormal vascular structure in multiple organs and altered blood pressure depending on their host environment. In this review, the endogenous role of AHR in cardiovascular physiology, including heart function, vascular development and blood pressure regulation has been summarized and discussed.

Key words: Aryl hydrocarbon receptor, blood pressure, cardiac hypertrophy, hypertension, hypotension

INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a transcription factor that belongs to the basic helix-loop-helix /PER-ARNT-SIM family of DNA binding proteins. There are two major categories of environmental compounds that activate AHR signaling: halogenated aromatic hydrocarbons (HAH), such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAH), such as benzo(*a*)pyrene. Unliganded AHR forms a complex including two copies of 90kD a heat shock protein (HSP90), one X-associated protein (XAP), and one p23 molecular chaperone protein in the cytoplasm.[1-4] After being activated by its ligands, cytoplasmic AHR translocates into the nucleus, disassociates from the chaperone complex, dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and transactivates target genes through binding to dioxin response elements (DRE) in promoter regions. AHR target genes include Phase I and Phase II metabolic enzymes, such as cytochrome P450 1A1 (CYP1A1), cytochrome P450 1B1 (CYP1B1), NAD(P)H: Quinone oxidoreductase I (NQO1) and aldehyde dehydrogenase 3 (ALHD3A1) [Figure 1]. The induction of xenobiotic metabolizing enzymes following AHR activation
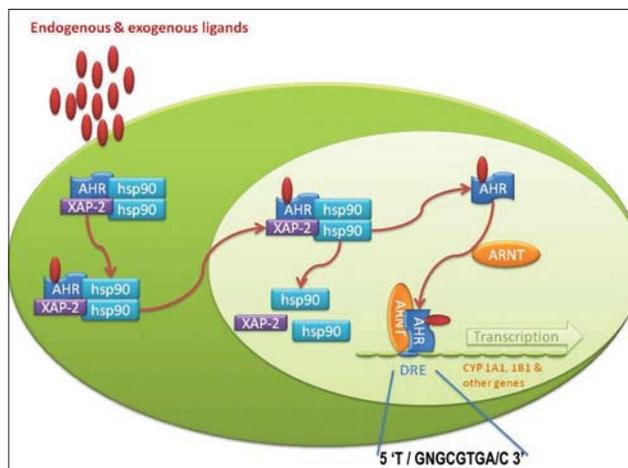
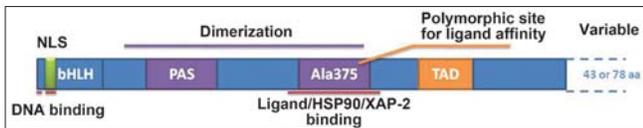**Figure 1:** Aryl hydrocarbon receptor signaling pathway

Figure 2: Aryl hydrocarbon receptor structure

is considered, at least in part, an adaptive response of the organism to its environment, which could decrease the potential toxicity of foreign chemicals. On the other hand, activation of AHR also mediates the toxicity of its environmental ligands.

The AHR molecule varies significantly across species in mediating TCDD toxicity,[5] as well as in molecular weight by almost 30kD, which is primarily due to the different positions of the translational termination codon.[6] Four murine AHR alleles, AHR^{b-1}, AHR^{b-2}, AHR^{b-3} and AHR^d, have been found and cloned from different inbred and wild mouse strains.[6-9] The AHR^d receptor has a lower ligand-binding affinity compared to the AHR^{b-1} and AHR^{b-2} alleles.[9] The AHR^{b-1} allele encodes a protein of 805 amino acids, the AHR^{b-2} and AHR^d alleles encode proteins of 848 amino acids, and the AHR^{b-3} allele encodes a protein of 883 amino acids. All proteins of the four alleles contain a basic helix-loop-helix motif (bHLH), PER-ARNT-SIM (PAS) domain and a transactivation domain (TAD), and their varied amino acids exist at the carboxyl end.[6] [Figure 2]. Human AHR is identical to mouse AHR at N-terminus and has 60% identity with mouse AHR at C-terminus.[6] An Ala$_{375}$→Val$_{375}$ polymorphism is responsible for the reduced ligand-binding affinity of the AHR^d receptor compared with the AHR^{b-2} receptor in both rodent and human.[9,10]

Through evolution of multicellular organisms, the function of AHR in environmental adaption has also been put to use in important physiological processes. AHR mRNA is expressed in multiple human tissues, with the highest expression in the placenta, relatively high expression in the lung, heart, pancreas and liver, and lowest expression in the kidney, brain and skeletal muscle.[11] Its mRNA has also been detected in multiple vascular beds in human, including pulmonary microvasclature, aortic arch and umbilical vein.[12,13] In the absence of exogenous ligands, the intrinsic activity of AHR signaling is subject to regulation by either endogenous ligands, including 2-(1'H-indole-3'-carbonyl)-thiazole- 4-carboxylic acid methyl ester, arachidonic acid metabolites, such as prostaglandinG2 and lipoxin4A, and heme metabolites, such as bilirubin; or nonligand activators, such as shear stress, cAMP and modified low-density lipoprotein (LDL).[14-22] Although none of these factors have been proved as high-affinity physiological activators of AHR, the endogenous function of AHR signaling, including heart function, vascular development and blood

pressure regulation, has been characterized using *ahr* gene-deficient mice. Due to the nature of AHR, a mediator of xenobiotics and a potential target in genetic modification of cardiovascular function, in this review, the function of this receptor in the cardiovascular system is summarized and discussed, which may shed light on the development of a new therapeutic methodology in cardiovascular disease prevention and treatment.

ARYL HYDROCARBON RECEPTOR IN HEART FUNCTION

In the 1990s, AHR-deficient mice were developed independently in three labs, by either deleting exon 1[23,24] or exon 2[25] of the gene. All three AHR-null mice had a mixed C57BL/6 ×129 background and displayed a slower growth rate within the first few weeks after birth, TCDD resistance, failure of xenobiotic CYP1A1 and CYP1A2 induction, maintained but decreased fertility and liver pathology.

The function of endogenous AHR signaling in heart development and physiology remained contradictory. AHR-deficient mice develop cardiac hypertrophy and fibrosis in adulthood with a sophisticated mechanism.[26-28] Early characterization of the enlarged heart in AHR-null mice suggested that enhanced vascular endothelial growth factor (VEGF) expression may contribute to the hypertrophy phenotype.[27] In 2003, Vasquez *et al*., reported increased size of cardiomyocytes and an anatomic remodeling without typical features of molecular remodeling, which was not consistent with hypertrophic growth secondary to pressure or volume overload.[28] This suggested an intrinsic role of AHR in cardiomyocyte size control. In the same year, Lund *et al*., indicated that cardiac hypertrophy in AHR-null mice was associated with high systemic arterial blood pressure as well as increased circulating angiotensin II (Ang II) and plasma endothelin-1 (ET-1) level.[29] This cardiac hypertrophic phenotype was primarily mediated by elevated circulating ET-1, thus treatment with BQ-123, an ET_A receptor antagonist, significantly attenuated the phenotype as well as the mRNA expression of cardiac hypertrophy markers, atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC).[30] Cardiac fibrosis were observed by both groups in AHR-null mice, suggesting a functional remodeling of the heart. A further study on altitude acclimated low blood pressure AHR knockout mice revealed that the hypertrophied heart is more likely a compensatory physiological effect to increase cardiac output in an attempt to increase blood pressure.[31] This is consistent with the absence of pathological cardiac hypertrophy markers reported by Vasquez *et al*.[28] A

recent study on AHR-null mice also indicated cardiac hypertrophy and fibrosis, which might involve Vav3, an activator of Rho/Rac GTPases, regulated by AHR.[32] The authors also demonstrated a thickening of arterial media wall and increased number of vascular smooth muscle cells in arterial walls. All the research data above, although inconsistently, suggest that local AHR signaling contributes to the development of cardiac hypertrophy and fibrosis that reflects a cardiac functional remodeling.

ARYL HYDROCARBON RECEPTOR IN VASCULAR DEVELOPMENT

The role of endogenous AHR in vascular development is also uncovered from the research on AHR knockout mice, which exhibit a spectrum of hepatic defects, including portal fibrosis and a smaller liver. The mechanism underlying the liver defect seems due to fetal hepatic necrosis caused by compromised perfusion,[33,34] and partially resulted from a patent ductus venosus in adulthood, which is mediated by loss of AHR in endothelial cells specifically.[34-36] Abnormal vascular structures have also been reported in the liver, kidney and hyaloid of AHR knockout mice.[35] A further investigation showed that the mice carrying the hypomorphic AHR allele also develop patent ductus venosus, which could be rescued by TCDD treatment.[34] In addition, nuclear translocation and DNA binding abilities of AHR are both required in the closure of ductus venosus, suggesting a transactivation mechanism in this particular endogenous AHR function.[37,38] Taken together, these two models suggest that the endogenous and exogenous ligand-activated AHR signaling may share the same signal transduction mechanism in mediating vascular development.

ARYL HYDROCARBON RECEPTOR IN BLOOD PRESSURE REGULATION

The role of the AHR agonist, TCDD, in inducing high blood pressure has been demonstrated in both epidemiology studies and research using mouse models, in which AHR-mediated cytochrome P450 overexpression may be involved.[39-43] Due to the similarity between endogenous and exogenous AHR signaling, it is not surprising that endogenous AHR also contributes to blood pressure regulation in addition to the cardiovascular development mentioned above. Anesthetized AHR-null mice were first found hypotensive in the absence of a heart rate difference at eight months of age.[28] The authors also reported a decreased cardiac output caused by diminished stroke volume in four-month-old AHR knockout mice.[28] This

finding suggested a role of AHR in causing hypotension by decreasing cardiac function. Later in the same year, Lund *et al*., reported high blood pressure in conscious AHR-null mice, associated with elevated circulating Ang II and ET-1 levels.[29] In this study, angiotensin converting enzyme blockade by captopril attenuated, but did not normalize elevated arterial blood pressure. Subsequently, ET-1 was identified as the primary factor causing high arterial blood pressure in those AHR-null mice.[30] Treatment with BQ-123, an ET_A receptor blocker, dramatically attenuated mean arterial blood pressure as well as plasma Ang II levels in AHR-null mice, suggesting increased Ang II as a secondary effect of ET-1 elevation. Another group also reported elevated arterial blood pressure in AHR-null mice, which was normalized by captopril treatment.[44] Their model also suggested an increase of vascular α-1D adrenoceptor expression that was involved in the hypertensive phenotype. Interestingly, both groups reported hypertension in AHR-null mice located at mild high altitude (Albuquerque NM, 1620m; Mexico City, 2240m). A further investigation of blood pressure in AHR-null mice indicated that loss of endogenous AHR signaling in mice led to hypotension at sea level and hypertension at mild high altitude, which was caused by different atmospheric oxygen levels.[12]

A recent study performed by the group in Albuquerque comprehensively investigated the role of AHR in blood pressure regulation using AHR heterozygous and null mice.[31] Their up-to-date data indicated a very interesting phenotype of AHR-null mice. After living for a few years at high altitude, the AHR null mice have a hypotensive phenotype, which mimics the blood pressure phenotype observed at sea level. Additionally, the former proposed mediators of high blood pressure, including high circulating Ang II and ET-1 levels, no longer occur in these animals. This suggests that the AHR-null mice in Albuquerque have physiologically adapted to the altitude and exhibit a blood pressure phenotype consistent with sea level animals. The hypotensive AHR-null mice exhibit a significantly higher level of endothelial nitric oxide synthase (eNOS) and enhanced vascular nitric oxide (NO) production compared to both wild-type and AHR heterozygous mice, which both have normal blood pressure. However, this is not likely the cause of hypotension in AHR-null mice, since N^ω-nitro-L-arginine (LNNA), a non-selective nitric oxide synthase (NOS) blocker, failed to normalize the blood pressure. Moreover, neither prazosin, an alpha1 adrenoceptor antagonist, nor hexamethonium, a ganglionic blocker treatment, causes any differences in the blood pressure change among AHR wild-type, heterozygous and null mice, suggesting an intact sympathetic activity in the blood pressure regulation of AHR-null mice. However, a research

group in Spain, Salamanca (802 m) compared AHR-null and Vav3-null mice, which developed similar cardiovascular remodeling and blood pressure, and suggested that the hypertension of AHR-null mice is mediated by Vav3 through a sympathoexcitation mechanism.[32] Although the role of AHR in blood pressure regulation remains to be elucidated, there is no doubt that AHR could serve as a target in the treatment of high blood pressure and other NO-dependent vascular diseases.

CONCLUSIONS

Most cardiovascular diseases are attributed to long term, repeated functional interruption and deposition of harmful factors in the cardiovascular system. The role of AHR in mediating xenobiotics-induced vascular damage has been well documented. However, the research results on the role of endogenous AHR in vascular homeostasis and blood pressure regulation still remain contradictory. From all the research on *ahr* gene-deficient mice, there is no doubt that AHR is one of the most important factors in maintaining blood pressure stability in those animals. The mechanism of more than 90% of the cases of human hypertension is unknown and ahr gene polymorphism has been detected in humans.[45] Therefore epidemiology research to correlate ahr gene polymorphism, altitude of residence and blood pressure phenotype will provide valuable insight into the role of AHR in human blood pressure control. On the other hand, endothelium-derived nitric oxide production is a critical prognostic parameter in vascular function. Further understanding of the role of AHR in NO generation in vasculature endothelium and vascular remodeling will also contribute to the prevention of vascular diseases, such as atherosclerosis. The nature of cardiovascular diseases suggests a multifactorial etiology and a long-lasting disease development process. The endogenous AHR signaling represents a very promising target for cardiovascular disease prevention and treatment due to its role in heart and vascular physiology, blood pressure regulation and vascular NO generation. Thus, the AHR function in the cardiovascular system requires careful and comprehensive investigation with employment of true littermate animals with a pure genetic background and well-controlled animal husbandry environment.

REFERENCES

1. Carver, LA, LaPres, JJ, Jain S, Dunham EE, Bradfield CA. Characterization of the Ah receptor-associated protein, ARA9. J Biol Chem 1998; 273:33580-7.

2. Kazlauskas A, Poellinger L, Pongratz I. Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon) receptor. J Biol Chem 1999; 274:13519-24.

3. Ma Q, Whitlock JP Jr. A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Biol Chem 1997; 272:8878-84.

4. Meyer BK, Pray-Grant MG, Vanden Heuvel JP, Perdew GH. Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. Mol Cell Biol 1998;18:978-88.

5. Poland A, Knutson JC. 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. Annu Rev Pharmacol Toxicol 1982;22:517-54.

6. Schmidt JV, Bradfield CA. Ah receptor signaling pathways. Annu Rev Cell Dev Biol 1996;12:55-89.

7. Poland A, Glover E, Taylor BA. The murine Ah locus: A new allele and mapping to chromosome 12. Mol Pharmacol 1987;32:471-8.

8. Poland A, Glover E. Characterization and strain distribution pattern of the murine Ah receptor specified by the Ahd and Ahb-3 alleles. Mol Pharmacol 1990;38:306-12.

9. Poland A, Palen D, Glover E. Analysis of the four alleles of the murine aryl hydrocarbon receptor. Mol Pharmacol 1994;46:915-21.

10. Ema M, Ohe N, Suzuki M, Mimura J, Sogawa K, Ikawa S *et al*. Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors. J Biol Chem 1994;269:27337-43.

11. Dolwick KM, Schmidt JV, Carver LA, Swanson HI, Bradfield CA. Cloning and expression of a human Ah receptor cDNA. Mol Pharmacol 1993;44:911-7.

12. Lund AK, Agbor LN, Zhang N, Baker A, Zhao H, Fink GD, et al. Loss of the aryl hydrocarbon receptor induces hypoxemia, endothelin-1, and systemic hypertension at modest altitude. Hypertension 2008;51:803-9.

13. Zhang N, Walker MK. Crosstalk between the aryl hydrocarbon receptor and hypoxia on the constitutive expression of cytochrome P4501A1 mRNA. Cardiovasc Toxicol 2007;7:282-90.

14. Henry EC, Bemis JC, Henry O, Kende AS, Gasiewicz TA. A potential endogenous ligand for the aryl hydrocarbon receptor has potent agonist activity *in vitro and in vivo*. Arch Biochem Biophys 2006;450:67-77.

15. Seidel SD, Winters GM, Rogers WJ, Ziccardi MH, Li V, Keser B, *et al*. Activation of the Ah receptor signaling pathway by prostaglandins. J Biochem Mol Toxicol 2001;15:187-96.

16. Schaldach CM, Riby J, Bjeldanes LF. Lipoxin A4: A new class of ligand for the Ah receptor. Biochemistry 1999;38:7594-600.

17. Sinal CJ, Bend JR. Aryl hydrocarbon receptor-dependent induction of cyp1a1 by bilirubin in mouse hepatoma hepa 1c1c7 cells. Mol Pharmacol 1997;52:590-9.

18. Mufti NA, Bleckwenn NA, Babish JG, Shuler ML. Possible involvement of the Ah receptor in the induction of cytochrome P-450IA1 under conditions of hydrodynamic shear in microcarrier-attached hepatoma cell lines. Biochem Biophys Res Commun 1995;208:144-52.

19. Mufti NA, Shuler ML. Induction of cytochrome P-450IA1 activity in response to sublethal stresses in microcarrier-attached Hep G2 cells. Biotechnol Prog 1995;11:659-63.

20. Eskin SG, Turner NA, McIntire LV. Endothelial cell cytochrome P450 1A1 and 1B1: Up-regulation by shear stress. Endothelium 2004;11:1-10.

21. McMillan BJ, Bradfield CA. The aryl hydrocarbon receptor is activated by modified low-density lipoprotein. Proc Natl Acad Sci USA 2007;104:1412-7.

22. Oesch-Bartlomowicz B, Huelster A, Wiss O, Antoniou-Lipfert P, Dietrich C, Arand M, *et al*. Aryl hydrocarbon receptor activation by cAMP vs. dioxin: Divergent signaling pathways. Proc Natl Acad Sci USA 2005;102:9218-23.

23. Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, *et al*. Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. Science 1995;268:722-6.

24. Mimura J, Yamashita K, Nakamura K, Morita M, Takagi TN, Nakao K, *et al*. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells 1997;2:645-54.

25. Schmidt JV, Su GH, Reddy JK, Simon MC, Bradfield CA. Characterization of a murine Ahr null allele: Involvement of the Ah receptor in hepatic growth and development. Proc Natl Acad Sci USA 1996;93:6731-6.

26. Fernandez-Salguero PM, Ward JM, Sundberg JP, Gonzalez FJ. Lesions

of aryl-hydrocarbon receptor-deficient mice. Vet Pathol 1997;34:605-14.

27. Thackaberry EA, Gabaldon DM, Walker MK, Smith SM. Aryl hydrocarbon receptor null mice develop cardiac hypertrophy and increased hypoxia-inducible factor-1alpha in the absence of cardiac hypoxia. Cardiovasc Toxicol 2002;2:263-74.

28. Vasquez A, Atallah-Yunes N, Smith FC, You X, Chase SE, Silverstone AE, et al. A role for the aryl hydrocarbon receptor in cardiac physiology and function as demonstrated by AhR knockout mice. Cardiovasc Toxicol 2003;3:153-63.

29. Lund AK, Goens MB, Kanagy NL, Walker MK. Cardiac hypertrophy in aryl hydrocarbon receptor null mice is correlated with elevated angiotensin II, endothelin-1, and mean arterial blood pressure. Toxicol Appl Pharmacol 2003;193:177-87.

30. Lund AK, Goens MB, Nunez BA, Walker MK. Characterizing the role of endothelin-1 in the progression of cardiac hypertrophy in aryl hydrocarbon receptor (AhR) null mice. Toxicol Appl Pharmacol 2006;212:127-35.

31. Zhang N, Agbor LN, Scott JA, Zalobowski T, Elased KM, Trujillo A, et al. An activated renin-angiotensin system maintains normal blood pressure in aryl hydrocarbon receptor heterozygous mice but not in null mice. Biochem Pharmacol 2010; 80:197-204.

32. Sauzeau V, Carvajal-Gonzalez JM, Riolobos AS, Sevilla MA, Menacho-Marquez M, Roman AC, et al. Transcriptional factor aryl hydrocarbon receptor (Ahr) controls cardiovascular and respiratory functions by regulating the expression of the Vav3 proto-oncogene. J Biol Chem 2011; 286:2896-909.

33. Harstad EB, Guite CA, Thomae TL, Bradfield CA. Liver deformation in Ahr-null mice: Evidence for aberrant hepatic perfusion in early development. Mol Pharmacol 2006;69:1534-41.

34. Walisser JA, Bunger MK, Glover E, Bradfield CA. Gestational exposure of Ahr and Arnt hypomorphs to dioxin rescues vascular development. Proc Natl Acad Sci USA 2004;101:16677-82.

35. Lahvis GP, Lindell SL, Thomas RS, McCuskey RS, Murphy C, Glover E, et al. Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice. Proc Natl Acad Sci USA 2000;97:10442-7.

36. Walisser JA, Glover E, Pande K, Liss AL, Bradfield CA. Aryl hydrocarbon receptor-dependent liver development and hepatotoxicity are mediated by different cell types. Proc Natl Acad Sci USA 2005;102:17858-63.

37. Bunger MK, Moran SM, Glover E, Thomae TL, Lahvis GP, Lin BC, et al. Resistance to 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity and abnormal liver development in mice carrying a mutation in the nuclear localization sequence of the aryl hydrocarbon receptor. J Biol Chem 2003;278:17767-74.

38. Bunger MK, Glover E, Moran SM, Walisser JA, Lahvis GP, Hsu EL, et al. Abnormal liver development and resistance to 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in mice carrying a mutation in the DNA-binding domain of the aryl hydrocarbon receptor. Toxicol Sci 2008;106:83-92.

39. Pesatori AC, Zocchetti C, Guercilena S, Consonni D, Turrini D, Bertazzi PA. Dioxin exposure and non-malignant health effects: A mortality study. Occup Environ Med 1998;55:126-31.

40. Kim JS, Lim HS, Cho SI, Cheong HK, Lim MK. Impact of Agent Orange exposure among Korean Vietnam veterans. Ind Health 2003;41:149-57.

41. Kang HK, Dalager NA, Needham LL, Patterson DG Jr, Lees PS, Yates K, et al. Health status of Army Chemical Corps Vietnam veterans who sprayed defoliant in Vietnam. Am J Ind Med 2006;49:875-84.

42. Dalton TP, Kerzee JK, Wang B, Miller M, Dieter MZ, Lorenz JN, et al. Dioxin exposure is an environmental risk factor for ischemic heart disease. Cardiovasc Toxicol 2001;1:285-98.

43. Kopf PG, Huwe JK, Walker MK. Hypertension, cardiac hypertrophy, and impaired vascular relaxation induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin are associated with increased superoxide. Cardiovasc Toxicol 2008;8:181-93.

44. Villalobos-Molina R, Vazquez-Cuevas FG, Lopez-Guerrero JJ, Figueroa-Garcia MC, Gallardo-Ortiz IA, Ibarra M, et al. Vascular alpha-1D-adrenoceptors are overexpressed in aorta of the aryl hydrocarbon receptor null mouse: Role of increased angiotensin II. Auton Autacoid Pharmacol 2008;28:61-7.

45. Harper PA, Wong JY, Lam MS, Okey AB. Polymorphisms in the human AH receptor. Chem Biol Interact 2002;141:161-87.

Author Help: Online submission of the manuscripts

Articles can be submitted online from http://www.journalonweb.com. For online submission, the articles should be prepared in two files (first page file and article file). Images should be submitted separately.

1) **First Page File:**
   Prepare the title page, covering letter, acknowledgement etc. using a word processor program. All information related to your identity should be included here. Use text/rtf/doc/pdf files. Do not zip the files.

2) **Article File:**
   The main text of the article, beginning with the Abstract to References (including tables) should be in this file. Do not include any information (such as acknowledgement, your names in page headers etc.) in this file. Use text/rtf/doc/pdf files. Do not zip the files. Limit the file size to 1024 kb. Do not incorporate images in the file. If file size is large, graphs can be submitted separately as images, without their being incorporated in the article file. This will reduce the size of the file.

3) **Images:**
   Submit good quality color images. Each image should be less than **4096 kb (4 MB)** in size. The size of the image can be reduced by decreasing the actual height and width of the images (keep up to about 6 inches and up to about 1800 x 1200 pixels). JPEG is the most suitable file format. The image quality should be good enough to judge the scientific value of the image. For the purpose of printing, always retain a good quality, high resolution image. This high resolution image should be sent to the editorial office at the time of sending a revised article.

4) **Legends:**
   Legends for the figures/images should be included at the end of the article file.