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AhR modulates NADPH oxidase activity via direct transcriptional regulation of $p40^{phox}$ expression

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Running Title: AhR regulates $p40^{phox}$ gene.

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ABBREVIATIONS:

3MC, 3-methylcholantren; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; BaP, benzo[a]pyrene; ChIP, chromatin Immunoprecipitation; CYP, Cytochrome P450; DCFDA, dichlorodihydrofluorescein diacetate, acetyl ester; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx, glutathione peroxidase; LKO, liver specific knockout; LUC, luciferase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROS, reactive oxygen species; siRNA, small interfering RNA; SOD, superoxide dismutase; TBE, tris-borate-EDTA; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic response element

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Abstract

p40^{phox}, a member of the NADPH oxidase subunits, plays an important role in the regulation of NADPH oxidase activity and the subsequent production of reactive oxygen species (ROS). In this study, we show that mouse *p40^{phox}* is a novel transcriptional target of the aryl hydrocarbon receptor (AhR), known as a dioxin receptor or xenobiotic receptor, in the liver. Treatment of mice with 3-methylcholantren (3MC) increased p40^{phox} gene expression in the liver. However, this induction of p40^{phox} gene expression was diminished by the deletion of the AhR gene in the liver. Consist with the *in vivo* results, the expression of the p40^{phox} gene was increased in 3MC-treated Hepa1c1c7 cells in an AhR-dependent manner. In addition, promoter analysis established *p40^{phox}* as a transcriptional target of AhR. Studies using the RNAi technique revealed that p40^{phox} is involved in the increase of NADPH oxidase activity and the subsequent ROS production in AhR-activated Hepa1c1c7 cells. Consequently, the results obtained here may provide a novel molecular mechanism of ROS production by exposure to dioxins.

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Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds are environmental pollutants which produce a variety of biochemical and toxic effects, such as hepatocellular damage, thymic involution, epithelial hyperplasia, teratogenesis, immune suppression, wasting syndrome, proliferation and / or differentiation of various epithelial tissues and tumor promotion in experimental animals (Poland and Knutson, 1982; Safe, 1986; Landers and Bunce, 1991; Pohjanvitra, and Tuomisto, 1994) . Most, if not all, of these effects of dioxins are mediated by their binding to a soluble protein designated the Aryl hydrocarbon receptor (AhR) (Femandez-Salguero et al., 1995; Schmidt et al., 1996; Mimura et al., 1997). Upon binding to dioxins, AhR translocates from the cytoplasm to nucleus and forms a heterodimer with the AhR nuclear translocator (ARNT). The AhR/ARNT dimer binds to a specific response element termed the xenobiotic response element (XRE) that contains a core five nucleotides 5'–gcgtg-3' and regulates expression of several genes, including drug metabolic enzymes (Reyes et al., 1992; Hankinson, 1995; Schmidt and Bradfield, 1996).

Accumulating evidences indicate that reactive oxygen species (ROS) such as $O_2^{\cdot}$- and H_2O_2 are generated by exposure to dioxins (Senft et al., 2002; Lin et al., 2007). Although ROS is essential for host defense against bacterial infection in phagocytes and acts as an intracellular mediator in non-phagocytes (Bokoch and Knaus, 2003), overproduction of ROS causes oxidative stress followed by the development of chronic diseases (Poli and Parola, 1997; Parola and Robino, 2001). Therefore, ROS produced by exposure to dioxin is, in part, responsible for the development of toxicity of dioxins. Among the enzymes responsible for ROS production, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase plays an important role (Bokoch and Knaus, 2003). In human macrophages, an AhR-dependent increase of the NADPH oxidase activity has been reported (Pinel-Marte et al., 2009). Also, the AhR agonist treatment increases the NADPH oxidase activity and subsequent formation of ROS in liver, kidney, heart, aorta and breast carcinoma cells (Puntarulo et al., 1992; Schlezinger et al.,

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1999; Lin et al., 2007; Kopf et al., 2008). However, the mechanism by which the AhR agonist increases the NADPH oxidase activity remains unclear.

NADPH oxidase is composed of a b-type membrane-bound cytochrome (termed flavocytochrome B) consisting of $gp91^{phox}$ /Nox2 and $p22^{phox}$ and several soluble components: $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1/2 in phagocytes. In non-phagocytes, the NADPH oxidase complex has a flexible characterization in which $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ can be replaced with the family homologues Nox1-5, Noxo1 and Noxa1, respectively (Bokoch and Knaus, 2003; De Minicis and Brenner, 2007). Thus, $p40^{phox}$ is an indispensable soluble component of NADPH oxidase complex. Expression of $p40^{phox}$ is originally found in neutrophils, but it is now recognized that $p40^{phox}$ is expressed in several other tissues, such as the liver, lung and spleen (Mizuki et al., 1998; Paik et al., 2011; Levin et al., 2012). By exogenous stimulation, $p40^{phox}$ is translocated from the cytoplasm to membrane and associate with flavocytochrome B, leading to ROS formation (Wientjes et al., 1993; El Benna et al., 1999). The role of $p40^{phox}$ was established based on the results of "gain of function" and "loss of function" studies, which showed that expression of $p40^{phox}$ was sufficient to activate superoxide production in COS7 cells stably transfected with b558, $p47^{phox}$, $p67^{phox}$, and the Fc gamma IIA receptor. Fc gamma IIA-stimulated NADPH oxidase activity was abrogated by point mutations in $p40^{phox}$ (Suh et al., 2006). Neutrophils from $p40^{phox}$ KO mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing (Ellson et al., 2006). Collectively, these results indicate that $p40^{phox}$ is a regulatory factor for NADPH oxidase.

In this study, to understand the mechanism by which the activated-AhR regulates ROS production, the effects of AhR ligand treatment on the NADPH oxidase activity, gene expressions of the subunits, and ROS production were analyzed.

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Materials and Methods

Animals and Drug Treatment.

Liver-specific AhR-deficient mice (AhR LKO mice) were generated by breeding of $AhR^{flox/flox}$ mice with knockin C57BL/6J mice carrying the Cre recombinase gene driven by the albumin promoter (The Jackson Laboratory, Bar Harbor, ME). Littermates that were negative for the Cre transgenes ($AhR^{flox/flox}$) were used as experimental controls. Mice were maintained at 23±1°C with 50±10% relative humidity, under a standard 12-h light/dark cycle with free access to water and food. Age- and sex-matched 14-week-old male mice were used for all experiments. 3-methylcholantren (3MC) (20 mg/kg) or benzo[a]pyrene (BaP) (30 mg/kg) was given by oral gavage for three consecutive days (Sugihara et al., 2008; Fang and Zhang, 2010). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nihon University.

Protein carbonylation and Thiobarbituric acid reactive substances (TBARS).

The level of carbonylated proteins and TBARS in the liver tissue was measured by using a Protein Carbonyl Fluorometric Assay Kit (Cayman Chemical, Ann Arbor, MI) and a TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI), respectively.

NADPH Oxidase Activity.

NADPH oxidase activity was measured by lucigenin assay (Selemidis et al., 2007). Mouse liver tissue or Hepa1c1c7 cells were homogenized in 50 mM phosphate buffer (pH 7.0) containing 150 mM sucrose, 1 mM EDTA and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) with a dounce homogenizer, and the resulting homogenate was stored on ice until use. Protein concentrations were measured by the Bradford method (Bradford, 1976). The reaction was started by adding the homogenates (10 μg of protein) to an assay solution containing lucigenin (5 μM) and NADPH (100 μM), and photon emission was measured for 3 min in a luminometer.

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Gene expression (qRT-PCR).

Total RNA was extracted with RNAiso Plus (Takara Co., Ltd., Otsu) according to the manufacturer's instructions. The cDNA was synthesized from 1.0 μg of total RNA by reverse transcriptase (Wako Pure Chemical Industries, Ltd., Osaka). Aliquots of cDNA were amplified on Stratagene MX3000 real-time PCR System using the SYBR-Green PCR reagents (Promega Co., Madison, WI). The mRNA expression levels were normalized against the 36B4 and GADPH expression and presented as relative expression levels. The primer sequences used are listed in Supplementary Table 1.

Western Blot Analysis.

Liver tissue or cells were lysed in the commercial lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) containing 1 μM dithiothreitol and the protease inhibitor cocktail. Protein concentrations were measured by the method of Bradford (Bradford, 1976). The proteins were resolved on SDS-PAGE, transferred onto the membranes, and probed with the antibodies against p40 ^{phox} (EMD Millipore Corporation, Billerica, MA) or beta-actin (Cell Signaling Technology, Inc.). Immunoreactive proteins were visualized with ECL western blotting detection reagents from Thermo Fisher Scientific Inc. (Waltham, MA).

Cell Culture.

Hepa1c1c7 and HepG2 cells, obtained from European Collection of Cell Culture, were maintained in Minimum Essential Medium Alpha or Minimum Essential Medium Eagle medium supplemented with 10% fetal bovine serum, respectively.

Small Interfering RNA (siRNA).

Hepa1c1c7 cells were transfected with either control stealth siRNA or stealth siRNA targeted for the AhR, or p40^{phox} (Life Technologies, Grand Island, NY) by using an X-tremeGENE siRNA transfection reagent (Roche Diagnostics K. K., Tokyo) according to the manufacturer's instructions.

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Electrophoretic Mobility Shift Assay (EMSA).

Nuclear proteins were prepared from Hepa1c1c7 cells treated with DMSO or 3MC for 16h (Wada et al., 2006). Also, mouse AhR and Arnt proteins were prepared using the TNT *in vitro* transcription and translation system (Promega). The binding reactions were performed as previously described (Wada et al., 2008). Protein-DNA complexes were resolved by electrophoresis through 5% polyacrylamide gel in 0.5 x TBE at 4^{o}C. For oligonucleotide competition experiments, unlabeled oligonucleotides were added to the reaction mixture at 100-fold molar excess to the radio-labeled probe. EMSA probe sequences are labeled in the figures (Fig. 3B). In some experiments, nuclear extracts were incubated with the antibody (0.5μg) against the AhR or the normal IgG.

Chromatin Immunoprecipitation (ChIP) Assay.

ChIP assay was performed essentially as described previously (Wada et al., 2008). In brief, Hepa1c1c7 cells were treated with DMSO or 3MC (3μM) for 16 h, cross-linked with formaldehyde, and lysed. The obtained cell extracts were subjected to immunoprecipitation with anti-AhR antibody (Enzo Life Sciences, Farmingdale, NY). Parallel samples were incubated with normal IgG as a negative control. The following PCR primer were used:

p40^{phox} –113; 5’- TAAAACTGCTCTATGGTGGGTGGA -3’,

p40^{phox} +19; 5’-ACTTCCCAGCTGCACAGCAGT -3’,

p40^{phox} –5526; 5’- CTAGCATTGGGGAGATGTGGAA -3’,

p40^{phox} –5323; 5’- TGGTGCAATCTTCCCGACTCCA -3’,

CYP1A1 –866; 5’-GTTTCAAAATAATACATTCAGATCTT-3’,

CYP1A1 –745; 5’-AACAGGTAAAAGACTGATGGACAGGC-3’.

Plasmid Constructs and Cell Transfection.

The 5’ regulatory region (-2979 bp to +226 bp) of the mouse *p40^{phox}* gene was amplified by PCR using mouse genomic DNA as the PCR template and the following oligonucleotides: p40^{phox} -2979 5’-ACCTGGTCAGGTGGCCTAGA-3’ and p40^{phox} +226 5’-

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CGCAGATCTGGCTGTGGGTAGTCAGAAAAG -3'. The PCR-amplified fragment was cloned into the pGL3-basic vector (Promega). Site-directed mutagenesis was performed by the PCR overextension method and confirmed by DNA sequencing (Xie et al., 2000). Hepa1c1c7 cells were transfected into 48-well plates with FuGene HD (Promega) (Wada et al., 2008; Watabe et al., 2010). After 24 h of incubation, the transfection medium was replaced with fresh medium containing 3MC or DMSO. The cells lysates were extracted 16 h later and assayed with a dual luciferase reporter assay system (Promega). The pRL-tk vector (Promega), was used as a normalization control to correct for variable transfection efficiencies. All transfections were performed in triplicate.

Flow Cytometry Measurement of ROS.

The amount of ROS was measured by flow cytometric analysis of 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA)(Life Technologies)-stained cells. Hepa1c1c7 cells were incubated with CM-H_2DCFDA (5 μM) at 37°C in the dark. After 15 min, cells were harvested, collected, and washed once with PBS. The pellet was suspended in 500 μL PBS and fluorescence emission from the oxidized dye was detected at 525 nm by using a FC500 flow cytometer (Beckman Coulter Inc., Indianapolis, IN) and fluorescence microplate reader (BMG Labtech, Ortenberg, Germany). Each result was analyzed using CXP cytometer software (Beckman Coulter). The data are represented as the mean fluorescence intensity calculated by subtracting the value of intracellular fluorescence without CM-H_2DCFDA.

Statistical analysis.

When applicable, results are represented as the means ± SD. Statistical analysis was performed using Student's *t*-test for comparisons between the groups.

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Results

***Activation of the AhR increased NADPH oxidase activity* in vivo** – To determine whether the AhR agonists induce oxidative stress in the liver and, if so, whether the effect is mediated by the AhR, we first measured the level of the oxidative stress markers such as TBARS and protein carbonylation in mice treated with the AhR agonist. As shown in Fig. 1A, the AhR agonist treatment increased the level of TBARS and carbonlyated proteins in the livers of control mice (Fig. 1A). The increase of oxidative markers was not observed in the AhR LKO mice liver treated with the AhR agonists (Fig. 1A). Study of the gene expression revealed that the level of *Cyp1a1* mRNA, a well-characterized target gene of the AhR, was strongly induced by both 3MC and BaP in the livers of control mice, but not those of the AhR LKO mice (Supplemental Fig. 1). Then, to gain the insight by which the AhR agonist induces oxidative stress, NADPH oxidase activity in the liver was determined. The AhR agonist treatment significantly increased the NADPH activity in the liver of control mice (Fig. 1B). In the livers of the AhR LKO mice, the enzyme activity was slightly induced by the AhR agonist treatment but the degree was less than those in the control mice liver (Fig. 1B). Among the subunits of NADPH oxidase, the expression of $p40^{phox}$ was significantly increased in the livers of control mice treated with AhR agonists (Fig. 1C). The expression of other NADPH oxidase subunit genes exhibited no substantial induction by the AhR agonist treatment (Fig. 1C). In the AhR LKO mice, the induction of $p40^{phox}$ expression in the liver by the AhR agonist treatment was diminished (Fig. 1C). Expression of $p67^{phox}$ was reduced in the control mice liver and induced in AhR LKO mice by BaP treatment (Fig. 1C). However, the alternation of $p67^{phox}$ expression was only observed in BaP-treated mice but not in 3MC-treated mice (Fig. 1C). The induction of $p40^{phox}$ protein in the livers of control mice treated with the AhR agonists was also confirmed by western blot analysis (Fig. 1D). This induction of $p40^{phox}$ protein was not observed in the livers of AhR LKO mice (Fig. 1D). These results indicate that AhR is involved in the regulation of $p40^{phox}$ gene expression *in vivo*.

***Activation of AhR induced the expression of $p40^{phox}$* in vitro** – In the next set of experiments, AhR expression was knocked down in mouse hepatoma Hepa1c1c7 cells by an RNAi technique (Fig. 2A),

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and the effects of AhR agonist treatment on the expression of *p40^{phox}* were examined. The inferior AhR activity in the AhR-knockdown cells was confirmed by the reduction of the induced-*Cyp1a1* mRNA level in the 3MC-treated cells compared to that in control cells (Fig. 2B). Among the expression of NADPH oxidase subunits examined, only *p40^{phox}* expression was induced in the control cells by 3MC treatment, and this induction was reduced by the knockdown of AhR expression (Fig. 2B). Other AhR agonists such as β-naphthoflavone (β-NF) and BaP were also capable to induce *p40^{phox}* mRNA expression in an AhR-dependent manner (Fig. 2C). The expression of *p40^{phox}* was induced by 3MC in a dose- and time-dependent manner (Fig. 2D and 2E). An increase in *p40^{phox}* expression by the 3MC treatment was also observed at the protein level as judged by western blot analysis (Fig. 2F). In addition, the induction of *p40^{phox}* mRNA by AhR ligands was observed in the cells of a human originated hepatoma cell line, HepG2 (Fig. 2G).

p40^{phox} was a transcriptional target of the AhR - To understand the molecular mechanism of *p40^{phox}* induction by AhR, we cloned the 2.9-kb 5' flanking region of the mouse p40^{phox} gene. As shown in Fig. 3A, 3MC treatment increased the 2.9-kb p40^{phox} promoter activity as judged by reporter gene assay. Deletion analysis localized the AhR responsive region to -147 bp to +244 bp (Fig. 3A). Inspection of this region identified a putative XRE (-GCGTG-) at -39 to -35 bp (*p40^{phox}*/XRE) upstream of the transcription start site (Fig. 3B). EMSA revealed that incubation of the DNA probe containing *p40^{phox}*/XRE with nuclear extract from 3MC-treated cells resulted in the formation of a DNA/protein complex (Fig. 3B). This complex was not observed by the incubation with nuclear extract from untreated cells. The specificity of the complex formation was confirmed by competition experiments (Fig. 3B). Also, the complex was disrupted by the incubation with anti-AhR antibody but not with normal IgG (Fig. 3B). Furthermore, the *in vitro* synthesized AhR and Arnt specifically bound to the DNA probe containing *p40^{phox}*/XRE (Supplemental Fig. 2). Introduction of a mutation in this XRE resulted in loss of responsiveness to the AhR/Arnt and 3MC (Fig. 3C). The recruitment of the AhR to *p40^{phox}*/XRE on the genome was confirmed by ChIP assay. In this ChIP assay, XRE in mouse Cyp1A1 (-866 to -745) and a part of the p40^{phox} gene (-5526 to -5323), which lacks XRE, were used as

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positive and negative controls, respectively. As shown in Fig. 3D, 3MC treatment resulted in the recruitment of the AhR to the promoter region encompassing *p40^{phox}*/XRE.

Induction of p40^{phox} expression is involved in the increase of the NADPH oxidase activity and the ROS production in the AhR-activated cells– The results described above indicate that *p40^{phox}* is a direct transcriptional target of AhR. Therefore, to examine whether increased expression of *p40^{phox}* by the AhR agonist results in the activation of NADPH oxidase activity and the subsequent ROS production, the NADPH oxidase activity and ROS level in *p40^{phox}*-knockdown Hepa1c1c7 cells were determined. Consistent with the results of *in vivo* experiments (Fig. 1B), the NADPH oxidase activity in control siRNA transfected-cells was increased by the 3MC treatment (Fig. 4A and B). This induction of NADPH oxidase activity by 3MC treatment was not observed in *p40^{phox}*-knockdown cells, indicating that the activated-AhR increases NADPH activity via transcriptional regulation of *p40^{phox}* (Fig. 4B). Several studies have reported that AhR agonists increase ROS production (Puntarulo et al., 1992; Schlezinger et al., 1999; Lin et al., 2007; Kopf et al., 2008; Pinel-Marte et al., 2009). The present study also showed that AhR agonists increased the basal- and the PMA-induced ROS production (Fig. 4C). In addition to NADPH oxidase, several enzymes, including xanthine oxidase and uncoupled nitric oxide synthase (NOS), play a role in ROS production (Granger et al., 1988; Miller et al., 2008). Thus, to confirm that NADPH oxidase was indeed responsible for the elevation of ROS production in AhR agonist-treated cells, NADPH oxidase activity was inhibited by knockdown of *p40^{phox}* expression or apocynin (NADPH oxidase inhibitor), and the level of ROS in the AhR-activated cells was determined. Treatment with either apocynin or *p40^{phox}* siRNA suppressed the basal- and the 3MC-induced ROS production (Fig. 4D), suggesting that increase of ROS production by 3MC is partly mediated by NADPH oxidase. ROS level is maintained by the balance of formation and elimination (Rodriguez et al., 2005). Therefore, we also examined the effect of AhR agonist treatment on the expression of antioxidative enzymes. The activation of AhR had little effect on the expression levels of these anti-oxidative enzymes expression (Fig. 4E).

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Discussion

Several lines of evidence have suggested that oxidative stresses are involved in the toxic effects of dioxin, e.g., hepatic and extra-hepatic lipid peroxidation, increased DNA damage, decreased cellular membrane fluidity, etc. (reviewed in Stohs et al., 1990). Also, the results in this study revealed that the AhR is, at least partly, responsible for the induction of these oxidative stresses by dioxins (Fig. 1A). The adverse effects of oxidative stress are largely mediated by ROS. Among the enzymes regulating the ROS level, NADPH oxidase plays central roles in ROS production. In this study, we demonstrated that, both *in vivo* and *in vitro*, *p40^{phox}*, a subunit of NADPH oxidase, is a novel target gene of the AhR (Fig. 1-3). Elevation of the *p40^{phox}* expression level results in an increase of NADPH oxidase activity and the subsequent cellular ROS production in the AhR-activated cells (Fig. 4B-D). The results in Fig. 4D showed that apocynin treatment or knockdown of *p40^{phox}* expression significantly, but partly, reduced the level of ROS induced by 3MC. AhR agonist treatment increases ROS level by inducing several target genes including *Cyp1a1* (Morel et al., 1999; Beyer et al., 2008). Collectively, the results in this study indicated that *p40^{phox}* is one of these target genes, and the transcriptional regulation of this gene and the subsequent increase of NADPH activity are part of the key events in ROS production in the AhR-activated cells. In Fig. 1C, changes in *p67^{phox}* expression level was observed in BaP-treated mice liver. However, this was not observed in 3MC-treated mice liver, although both 3MC and BaP are capable to increase the NADPH activity (Fig. 1B and C). Thus alternation of *p67^{phox}* expression level in the BaP-treated mice liver is unlikely associated with elevation of NADPH activity. Also, activation of the AhR had no substantial effects on expression of anti-oxidant enzymes, indicating that elevation of ROS level by AhR-agonist treatment is mainly due to increase of the production (Fig. 4E).

Although it has long been recognized that the major cells expressing p40^{phox} are hematopoietic cells (Zhan et al., 1996), several recent studies have revealed that p40^{phox} is expressed in non-phagocytic cells and tissues such as the lung, liver, brain and spleen (Mizuki et al., 1998; Chessa et al., 2010; Paik et al., 2011). Here we showed the expression of p40^{phox} in the mouse liver. AhR LKO mice used in this study defect the AhR expression only in parenchymal cell but not in non-

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parenchymal cells (Walisser et al, 2005). Therefore, increases of *p40^{phox}* mRNA level and NADPH activity in the liver of mice treated with AhR agonists are most likely occurred in parenchymal cells, but not in kupffer cells or other non-parenchymal cells. As described above, NADPH oxidase is involved in the development of hepatic damages in response to the exposure to various chemicals including several environmental contaminants (Straub et al., 2008; Yang et al., 2008). Thus, the understanding of the transcriptional regulation of *p40^{phox}* expression by the AhR may provide the additional insight by which environmental contaminants increase cellular ROS level in the liver. However, to date, the limited information on transcriptional regulation of *p40^{phox}* in non-phagocytic cells and tissues are available. There has been a study which showed that PU.1, which is an ETS-domain transcription factor regulating in hematopoiesis, is involved in the transcriptional regulation of *p40^{phox}* in adipocytes as well as in neutrophils (Li et al., 1997; Suzuki et al., 1998; Li et al., 2002; Lin et al., 2012). Another report suggested that the AP2-binding site and NF-El consensus-binding site exist in the human p40^{phox} promoter region (Zhan et al., 1996). In addition to these findings, the present study revealed that the AhR binds to the putative XRE sequence located at -39/-35 bp of the mouse p40^{phox} promoter region and activates its promoter activity (Fig. 3). Therefore, the significance of the current findings is that this is the first report on the transcriptional regulatory mechanism of *p40^{phox}* expression in the mouse liver. It is also of interest that treatment with AhR ligands increased the expression of *p40^{phox}* in cell of a human originated hepatoma cell line, HepG2 (Fig. 2G). Within -1.2 kb upstream of the human p40^{phox} transcriptional start site, there are two putative XRE candidates (data not shown). Thus, certainly, one possible regulatory mechanism of *p40^{phox}* expression in humans is the direct activation of these XRE by the AhR. Alternatively, human p40^{phox} expression could be indirectly induced by the other AhR target genes or the non-genomic action of AhR ligands as exampled in Nrf2 or cytosolic phospholipase A2 expression (Miao et al., 2005; Dong et al., 2008;). A future promoter analysis of the human p40^{phox} gene would further reveal not only the regulatory mechanisms of p40^{phox} gene expression by the AhR but also the molecular mechanism by which AhR agonists induced NADPH oxidase activity and the subsequent ROS production in humans.

In summary, the present work revealed a novel function of AhR in the regulation of NADPH oxidase activity and the subsequent ROS production in the liver. It is well recognized that

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overproduction of ROS causes severe chronic diseases in the liver (Poli and Parola, 1997; Parola and Robino, 2001). Consequently, the results shown in this study lead us to speculate that development of a beneficial AhR antagonist could be useful for achieving therapeutic benefits in patients with chronic liver diseases.

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Authorship Contributions

Participated in research design: Taira Wada, Hiroshi Sunaga, and Shigeki Shimba.

Conducted experiments: Taira Wada, Hiroshi Sunaga, and Reiko Ohkawara.

Contributed new reagents or analytic tools: Taira Wada, Hiroshi Sunaga, and Reiko Ohkawara.

Performed data analysis: Taira Wada, Hiroshi Sunaga, Reiko Ohkawara, and Shigeki Shimba.

Wrote or contributed to the writing of the manuscript: Taira Wada, and Shigeki Shimba.

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Footnotes

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Figure Legends

Figure 1. Activation of the AhR induced NADPH oxidase activity and the expression of p40^{phox} in the mouse liver.

AhR $^{flox/flox}$ (control) mice and liver-specific AhR KO (AhR LKO) mice were treated with corn oil, 3MC (20mg/Kg), or BaP (30mg/Kg) for 3 consecutive days. A, levels of TBARS (left) and protein carbonlyation (right) in the livers of the control mice and AhR LKO mice. B, NADPH oxidase activity in the livers of the control mice and AhR LKO mice. The value of corn oil-treated control mice was normalized to 1. C, qRT-PCR analysis of NADPH oxidase subunit expression in the livers of control mice and AhR LKO mice. The value of corn oil-treated mice was normalized to 1. D, a representative western blot of p40^{phox} protein in the liver extract of mice treated with corn oil or AhR agonist. Samples were prepared from two different mice with each genotype.

The data represent the average and standard deviation from 3-4 mice per group.

*, P<0.05; **, P<0.01 compared to the corn oil (A) or the normalized control (B and C).

Figure 2. Activation of the AhR induced the expression of p40^{phox} in Hepa1c1c7 cells.

A - C, Hepa1c1c7 cells were transfected with control siRNA or siRNA specific for AhR.

A, qRT-PCR analysis of AhR expression in the siRNA-transfected cells. The value of control siRNA-transfected cells was normalized to 1. B, qRT-PCR analysis of NADPH oxidase subunit expression in the siRNA-transfected cells treated with DMSO or 3MC (3 μM) for 16 h. The value of DMSO-treated cells transfected with control siRNA was normalized to 1. C, qRT-PCR analysis of p40^{phox} expression in the siRNA-transfected cells treated with DMSO or AhR agonist (3 μM) for 16 h. D, qRT-PCR analysis of p40^{phox} expression in Hepa1c1c7 cells treated with DMSO or the indicated concentration of 3MC for 16 h. E, qRT-PCR analysis of the expression of p40^{phox} and Cyp1a1 in Hepa1c1c7 cells treated with 3MC (3 μM) for the indicated period of time. F, a representative western blot of p40^{phox} protein in Hepa1c1c7 cells treated with DMSO or 3MC (3 μM) for 16 h G, qRT-PCR analysis of p40^{phox} expression in HepG2 cells treated with DMSO or AhR agonist (3 μM) for 16 h. In C-E and G, The value of DMSO-treated cells was normalized to 1.

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The data represent the average and standard deviation from 3 independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001 compared to the normalized control (A, B, D, E) or to the control siRNA-transfected cells (C).

Figure 3. Activation of the AhR increased the mouse p40^{phox} promoter activity.

A, luciferase activity in DMSO- or 3MC (3 μM)-treated Hepa1c1c7 cells transfected with the reporter plasmids containing the p40^{phox} promoter. The value of DMSO-treated cells was normalized to 1. B, partial DNA sequences of the mouse p40^{phox} gene promoter. The putative p40^{phox}/XRE sequences are in capitals and the mutated nucleotides are underlined. EMSA binding reactions were performed by incubating the nuclear extracts with ^{32}P-labeled double-stranded oligonucleotides specifying the p40^{phox}/XRE. In the competition lanes, unlabeled probes were present in 100-fold molar excess relative to the radio-labeled probe. Anti-AhR antibodies or normal IgG were added to the performed DNA:protein complexes prior to electrophoresis. The arrow indicates the position of the AhR complex. C, luciferase activity in DMSO- or 3MC (3 μM)-treated Hepa1c1c7 cells transfected with the reporter plasmids containing the p40^{phox} promoter (-536 bp to +226 bp) or its mutant variant in the presence of AhR, Arnt or empty expression vector. The value of DMSO-treated cells transfected with pcDNA 3.1 and wild type promoter was normalized to 1. D, ChIP analysis of the interaction between AhR and the region containing the p40^{phox}/XRE in Hepa1c1c7 cells treated with 3MC (3 μM) for 16 h.

The data represent the average and standard deviation from 3 independent experiments.

***, P<0.001compared to the normalized control (A) or to the indicated reference (C).

Figure 4. Activation of the AhR increases NADPH oxidase activity and the subsequent ROS production in a p40^{phox}-dependent manner.

A and B, Hepa1c1c7 cells were transfected with control siRNA or two distinct siRNA specific for p40^{phox}. A, qRT-PCR analysis of p40^{phox} expression in the siRNA-transfected cells. B, NADPH oxidase activity in the siRNA-transfected cells. The value of control siRNA-transfected cells treated

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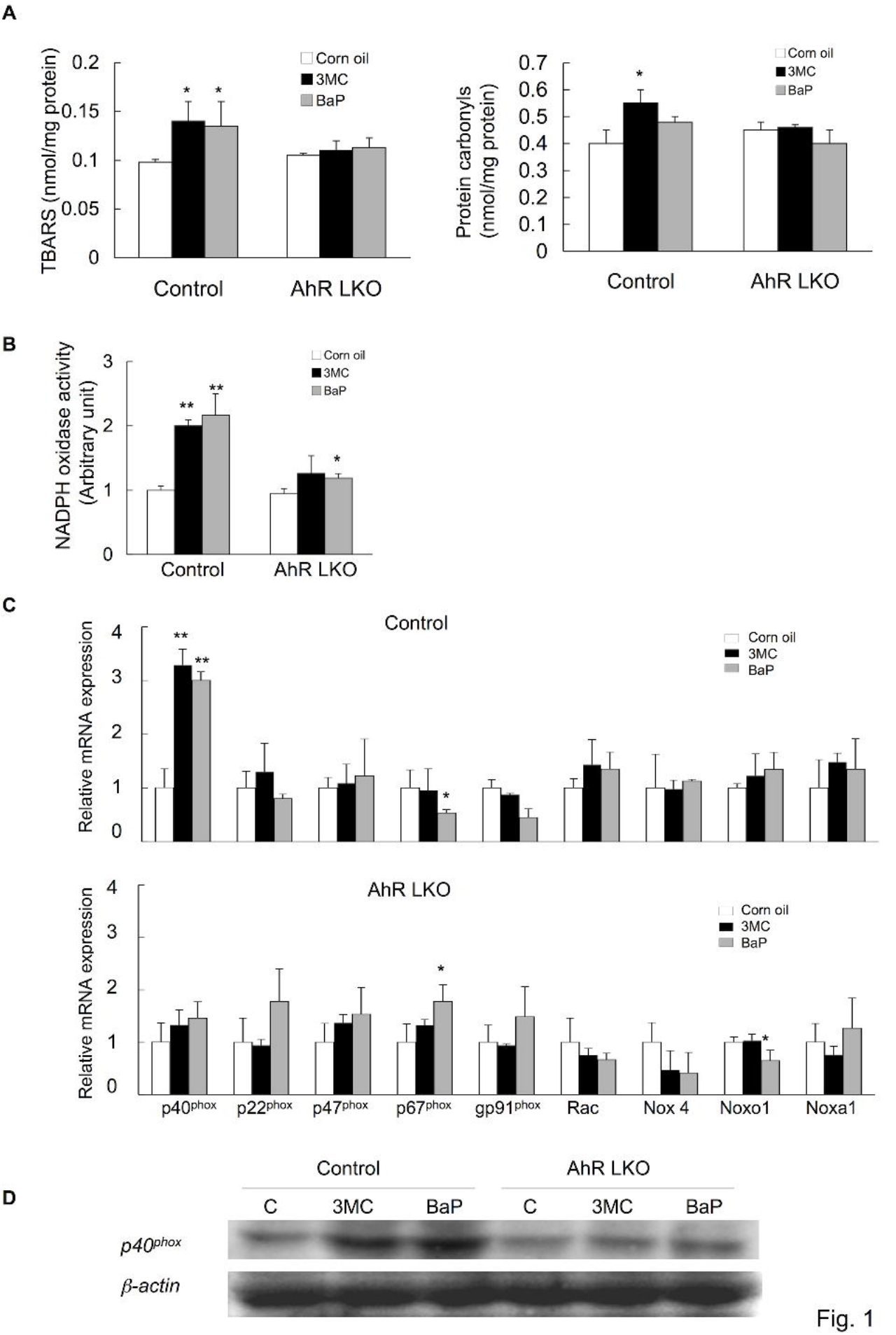
with DMSO was normalized to 1. C, ROS level measured by DCF fluorescence intensity in Hepa1c1c7 cells treated with DMSO, 3MC (3 μM), or BaP (3 μM) for 16 h. The cells were incubated in the presence or absence of PMA (1 μM) for 30 min and then treated with the fluorescence probe for an additional 15 min. The value of cells treated with DMSO in the absence of PMA was normalized to 1. D, ROS level measured by DCF fluorescence intensity in Hepa1c1c7 cells treated with DMSO or 3MC in the presence or absence of apocynin (100 nM) or an siRNA targeted for $p40^{phox}$. E, qRT-PCR analysis of the expression of anti-oxidative enzymes in Hepa1c1c7 cells treated with DMSO or 3MC. The value in control siRNA-transfected cells treated with DMSO was normalized to 1.

The data represent the averages and standard deviation from 3 independent experiments n=3-4 mice per each group. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared to the indicated reference (A, C, D) or to the normalized control (B).

Figure 5. Schematic representation of the molecular mechanism by which the AhR modulates hepatic NADPH oxidase activity and ROS productivity.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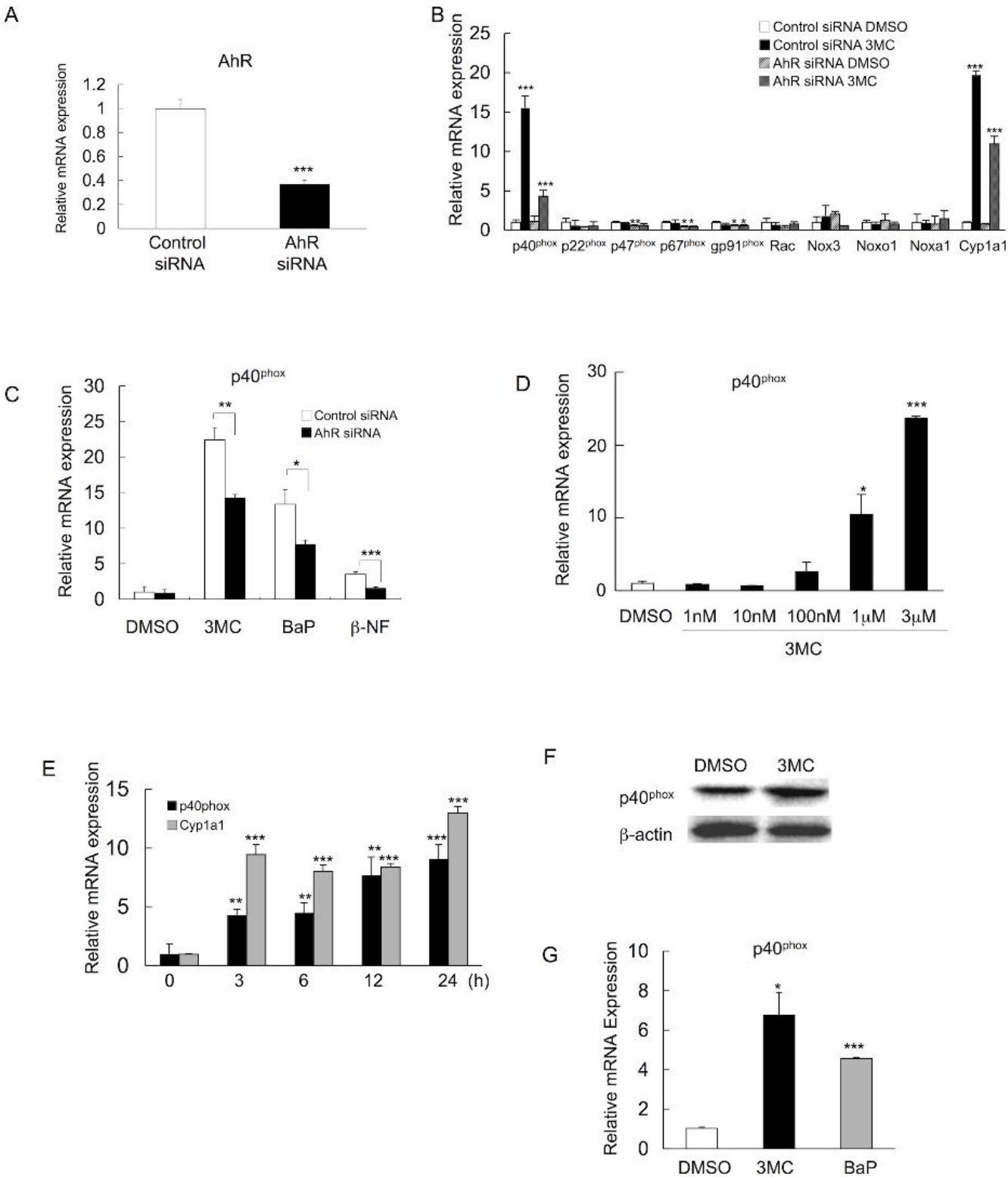
0.15
0.1
0.05
0
Corn oil
3MC
BaP
Control
AhR LKO
Protein carbonyls (nmol/mg protein)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
B
NADPH oxidase activity (Arbitrary unit)
3
2
1
0
C
Control
AhR LKO
Relative mRNA expression
4
3
2
1
0
p40phox
p22phox
p47phox
p67phox
gp91phox
Rac
Nox 4
Noxo1
Noxa1
D
C
3MC
BaP
p40phox
β-actin

Fig. 1


A
AhR
Relative mRNA expression
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1
0.8
0.6
0.4
0.2
0
***
Control siRNA
AhR siRNA
B
Relative mRNA expression
25
20
15
10
5
0
Control siRNA DMSO
Control siRNA 3MC
AhR siRNA DMSO
AhR siRNA 3MC
p40phox
p22phox
p47phox
p67phox
gp91phox
Rac
Nox3
Noxo1
Noxa1
Cyp1a1
C
p40phox
Relative mRNA expression
30
25
20
15
10
5
0
Control siRNA
AhR siRNA
DMSO
3MC
BaP
β-NF
D
p40phox
Relative mRNA expression
30
20
10
0
DMSO
1nM
10nM
100nM
1μM
3μM
3MC
E
Relative mRNA expression
15
10
5
0
p40phox
Cyp1a1
0
3
6
12
24 (h)
F
DMSO
3MC
p40phox
β-actin
G
p40phox
Relative mRNA Expression
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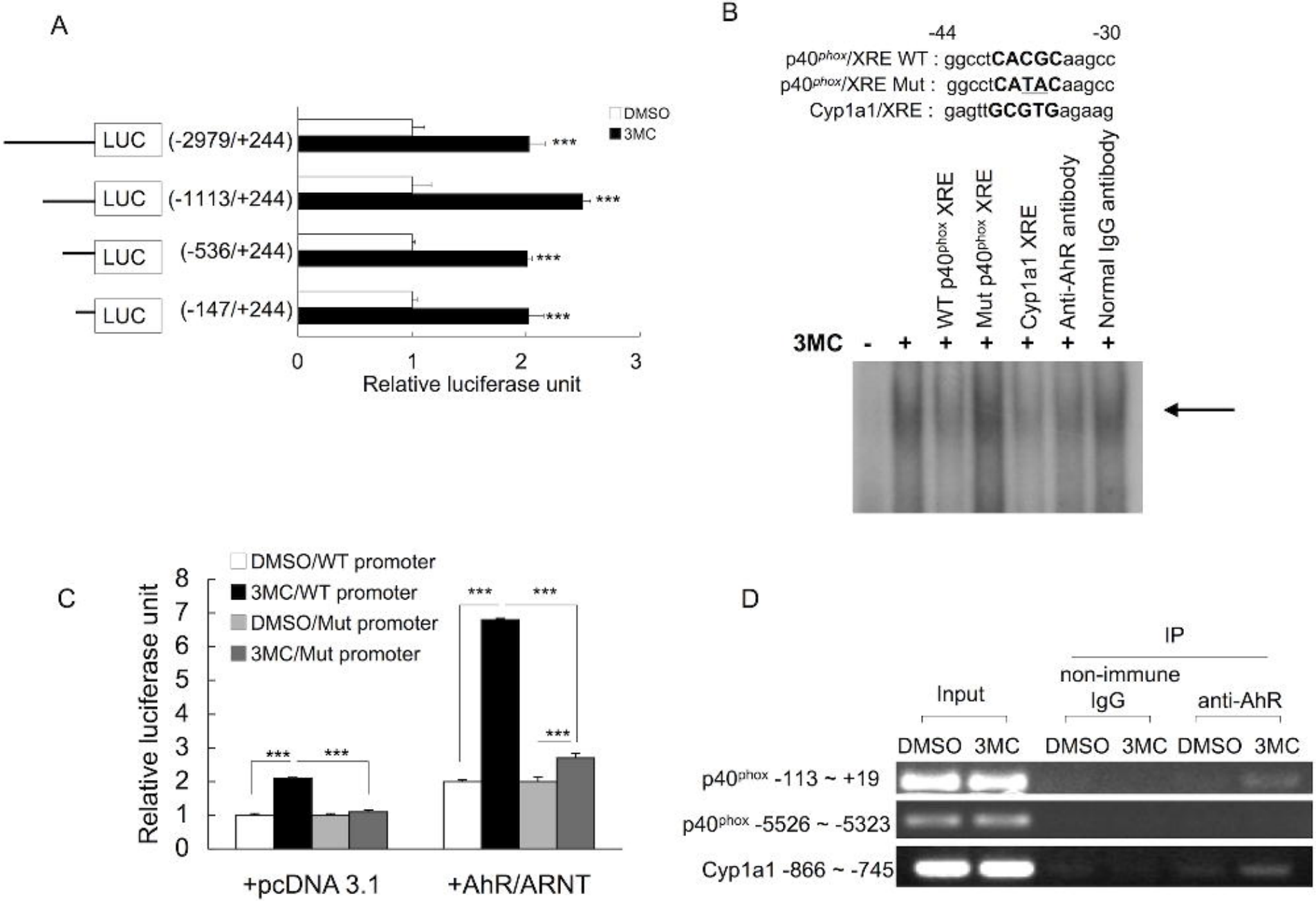
8
6
4
2
0
DMSO
3MC
BaP

Fig. 2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(-1113/+244)
(-536/+244)
(-147/+244)
DMSO
3MC
***
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2
3
Relative luciferase unit
B
-44
-30
p40phox/XRE WT : ggcctCACGCaagcc
p40phox/XRE Mut : ggcctCATACaagcc
Cyp1a1/XRE : gagttGCGTGagaag
3MC
-
+
+ WT p40phox XRE
+ Mut p40phox XRE
+ Cyp1a1 XRE
+ Anti-AhR antibody
+ Normal IgG antibody
C
DMSO/WT promoter
3MC/WT promoter
DMSO/Mut promoter
3MC/Mut promoter
Relative luciferase unit
0
1
2
3
4
5
6
7
8
***
+pcDNA 3.1
+AhR/ARNT
D
IP
Input
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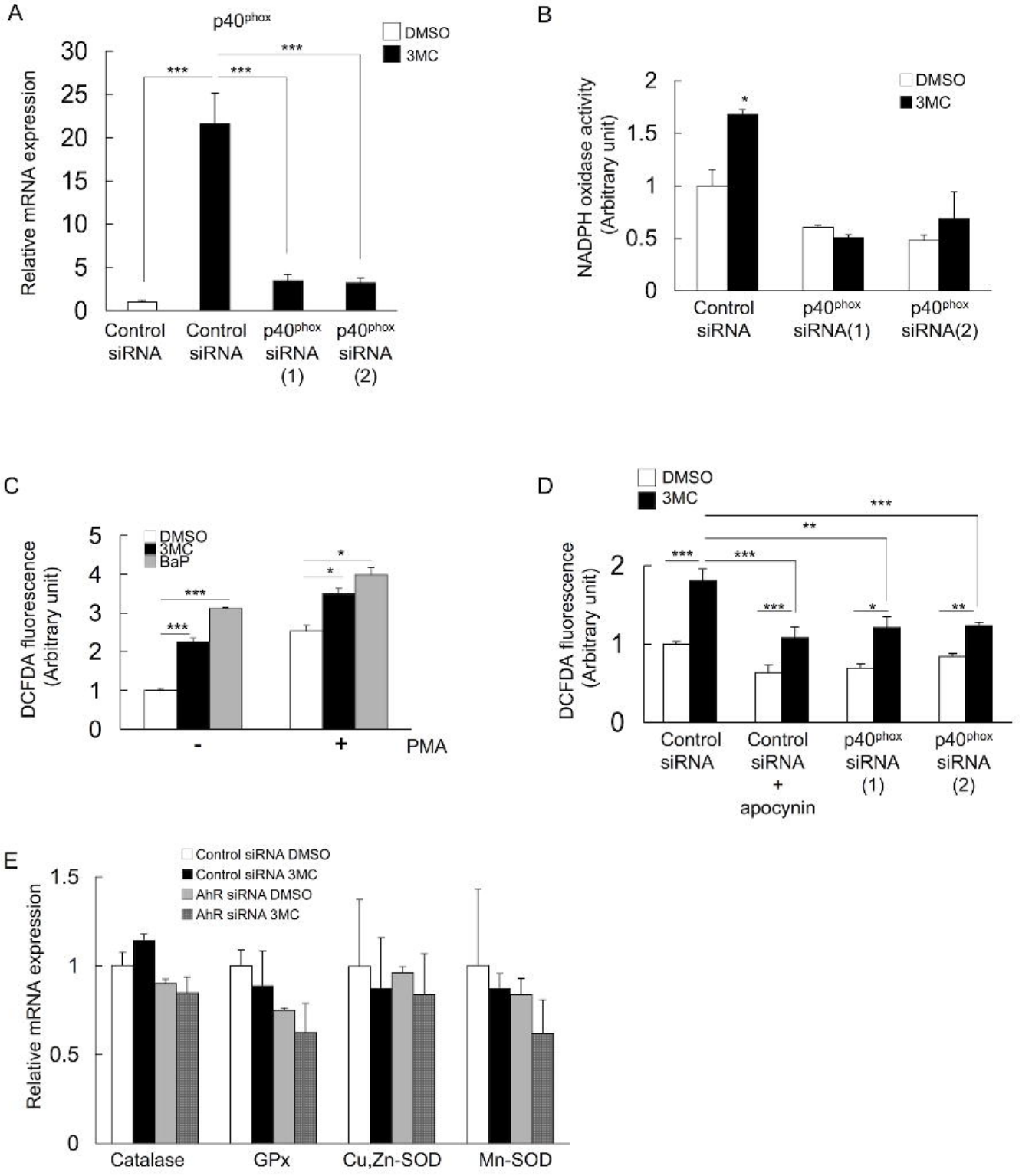
p40phox -5526 ~ -5323
Cyp1a1 -866 ~ -745

Fig. 3


A
p40phox
DMSO
3MC
Relative mRNA expression
Control siRNA
Control siRNA
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p40phox siRNA (2)
B
DMSO
3MC
NADPH oxidase activity (Arbitrary unit)
Control siRNA
p40phox siRNA(1)
p40phox siRNA(2)
C
DMSO
3MC
BaP
DCFDA fluorescence (Arbitrary unit)
PMA
D
DMSO
3MC
DCFDA fluorescence (Arbitrary unit)
Control siRNA
Control siRNA + apocynin
p40phox siRNA (1)
p40phox siRNA (2)
E
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Control siRNA 3MC
AhR siRNA DMSO
AhR siRNA 3MC
Relative mRNA expression
Catalase
GPx
Cu,Zn-SOD
Mn-SOD

Fig. 4

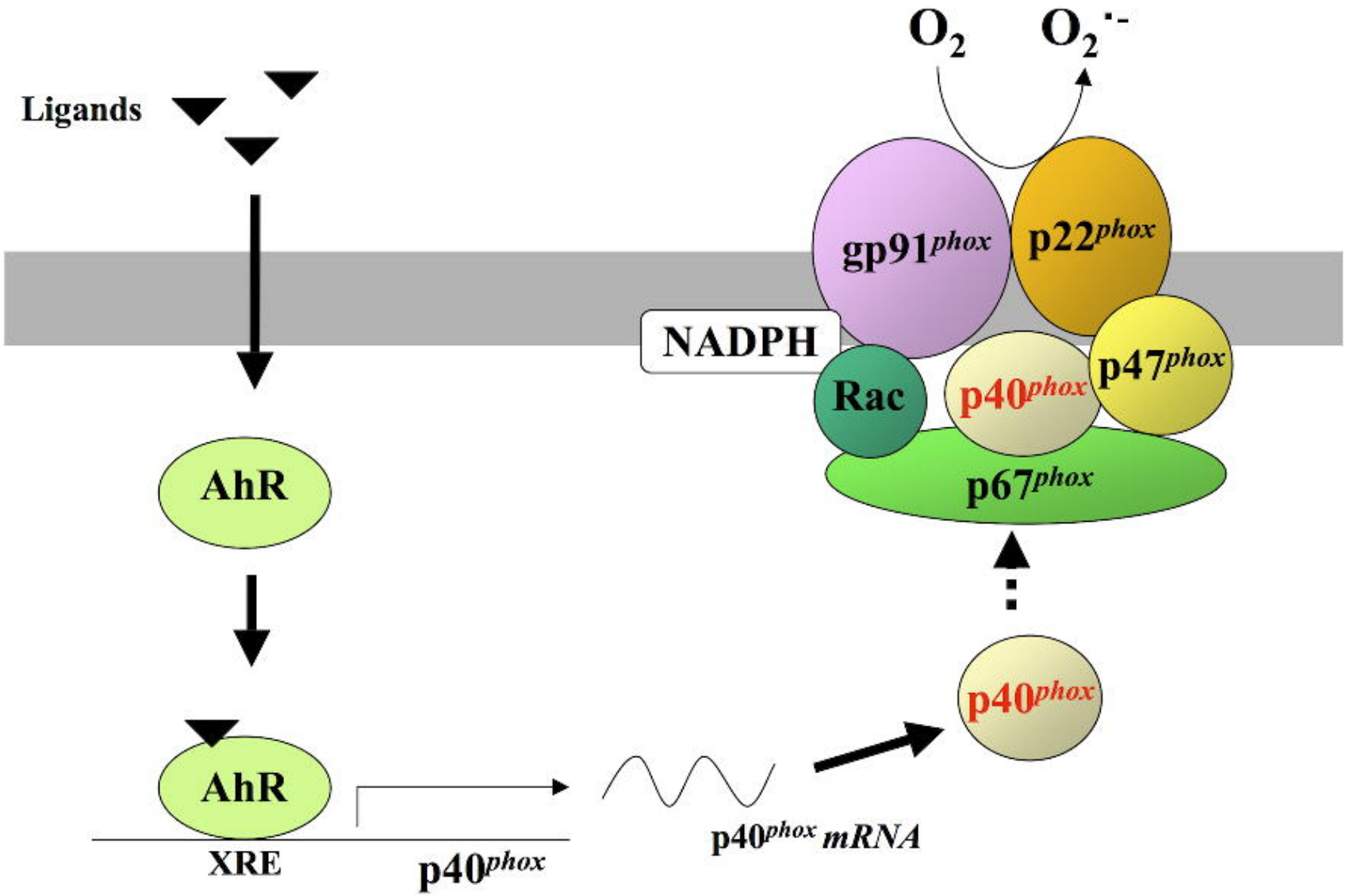
O_2
$O_2^{\cdot -}$
Ligands
gp91^{phox}
p22^{phox}
NADPH
Rac
p40^{phox}
p47^{phox}
p67^{phox}
AhR
AhR
XRE
p40^{phox}
p40^{phox} mRNA
p40^{phox}

Fig. 5