Endothelin-1–Mediated Increase in Reactive Oxygen Species and NADPH Oxidase Activity in Hearts of Aryl Hydrocarbon Receptor (AhR) Null Mice

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Received May 19, 2005; accepted August 9, 2005

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor characterized to play a role in detection and adaptation to environmental stimuli. Genetic deletion of the AhR results in cardiac hypertrophy that is mediated primarily by endothelin-1 (ET-1); ET-1 has been implicated in the elevation of reactive oxygen species (ROS) in the heart, which are thought to contribute to several cardiovascular disorders, including cardiac hypertrophy. Thus, we tested the novel hypothesis that ET-1 induces ROS in AhR null mice via ETA receptor activation. We first confirmed the presence of ROS in the hearts of AhR null mice by measuring superoxide (O2·−)-dependent oxidation of dihydroethidium. Ethidium fluorescence was increased 10-fold in the hearts of AhR null mice, compared to the wild type. Then, to elucidate whether ET-1 mediated the increase in ROS, mice were chronically treated with 100 ng/kg/day of the ETA receptor antagonist BQ-123. In AhR null mice, BQ-123 significantly reduced elevated plasma 8-isoprostane, a systemic end product of phospholipid oxidation by ROS, and cardiac thiobarbituric acid reactive substances (TBARS), a nonspecific assessment of ROS production. Furthermore, BQ-123 reduced both cardiac lucigenin chemiluminescence and cardiac mRNA expression of NAD(P)H oxidase subunits gp91phox, p47phox, and p67phox in AhR null mice below the levels observed in wild-type mice. These findings demonstrate that ET-1 activation of ETA receptors mediates an increase in ROS that is associated with cardiac hypertrophy in AhR null mice. In addition, the ET-1–mediated increase in ROS appears to be initiated via increased NAD(P)H oxidase activity.

Key Words: Aryl hydrocarbon receptor (AhR); reactive oxygen species; cardiac hypertrophy; endothelin-1; NAD(P)H oxidase.

INTRODUCTION

The AhR is a ligand-activated, cytosolic transcription factor belonging to the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) family of DNA-binding proteins. This protein family has been characterized to play a role in the detection and adaptation to environmental stimuli (Gu et al., 2000), including hypoxia. Although an endogenous ligand (Song et al., 2002) has recently been identified, the role of the AhR in normal physiological processes has not been fully elucidated. However, generation of the AhR null mouse has revealed definitive evidence for a role of the AhR in cardiovascular homeostasis. Genetic deletion of the AhR results in profound effects on the cardiovascular system that are characterized by hypertension and progressive cardiac hypertrophy (Fernandez-Salgueiro et al., 1997; Lund et al., 2003; Thackaberry et al., 2002; Vasquez et al., 2003) with significant increases in left ventricular mass, cardiac fibrosis, reduction of cardiac fractional shortening, and elevations in plasma angiotensin II (Ang II) and ET-1 (Lund et al., 2003). Although blockade of Ang II synthesis by treatment with an angiotensin-converting enzyme (ACE) inhibitor ameliorates the hypertension and cardiac hypertrophy, it does not normalize either to pretreatment values (Lund et al., 2003). In contrast, blockade of ET-1 activation of ETA receptors in AhR null mice normalizes the cardiac hypertrophy and nearly reduces mean arterial blood pressure (MAP) to wild-type values, suggesting that ET-1 is the primary mediator of the cardiovascular pathology (Lund et al., 2005).

Endothelin-1, a potent vasoactive and mitogenic peptide produced mainly by endothelial cells, is associated with initiation and progression of cardiac hypertrophy (Shubeita et al., 1990), mainly through ETA receptors on the myocardium. The role of ET-1 in cardiac hypertrophy has been confirmed through receptor antagonist studies, such that treatment results in attenuated progression of cardiac remodeling (Ehmke et al., 1999). Reactive oxygen species (ROS) have been shown to be critical mediators of ET-1-induced growth-promoting signaling events involved in the hypertrophic pathways in vascular smooth muscle cells (Daou and Srivastava, 2004) and cardiomyocytes (Hirotani et al., 2002). The role of ROS in ET-1–induced cardiac hypertrophy has been further confirmed through studies showing that ET-1–mediated generation of ROS in cardiac hypertrophy can be inhibited by pretreatment with an antioxidant (Xu et al., 2004). Although it is not yet
understood how an increase in ROS may contribute to ET-1–dependent cardiac hypertrophy, recent studies suggest that ROS modulates vascular tone in both arteries and veins, resulting in increased total peripheral resistance and elevated blood pressure (Thakali et al., 2005). Additionally, ROS may induce cardiac hypertrophy by activating signal transduction pathways, such as mitogen-activating protein kinase (MAPK), which mediate the hypertrophic response of cardiomyocytes (Cheng et al., 2005).

In mammalian tissues, ROS are formed under both physiological and pathological conditions. Myocardial ROS, specifically the superoxide anion (O$_2^-$), have been implicated in a large number of diseases, with evidence from both experimental and clinical studies suggesting a causal role of oxidative stress in the pathogenesis of congestive heart failure and cardiac hypertrophy (Dhalla et al., 2000; Sugden and Clerk, 1998). The role of ROS in cardiac hypertrophy is further supported by the finding that antioxidant treatment inhibits cardiac myocyte hypertrophy in both neonatal (Nakamura et al., 1998) and adult rat myocytes (Tanka et al., 2001).

Although the sources of ROS in the hypertrophying heart have not yet been fully elucidated, phagocyte-like NAD(P)H oxidases have emerged as a major source of ROS generation in the cardiovascular system (Li et al., 2002). The NAD(P)H oxidase complex consists of a core heterodimer comprised of a phagocytic oxidase (p22phox) subunit and a glycoprotein (gp91phox) subunit (or homologs termed Nox1 or Nox4), and four regulatory subunits: p47phox, p67phox, p40phox, and rac1. NAD(P)H oxidases have been identified in cardiomyocytes (Xiao et al., 2002), and subunits of the NAD(P)H oxidase complex have been found to be upregulated in the myocardium in states of cardiac pathology (Li et al., 2002).

It has not been determined whether an increase in ROS is associated with the ET-1–dependent cardiac hypertrophy in AhR null mice. Thus, we tested the novel hypothesis that increased ET-1 signaling via the ETA receptor induces ROS, specifically O$_2^-$, via NAD(P)H oxidase induction in the hearts of AhR null mice.

**MATERIALS AND METHODS**

**Animals and study protocols.** AhR null mice were obtained from Dr. Frank Gonzales (National Cancer Institute) and C57BL/6N AhR wild type were purchased from Harlan; all were maintained by the Animal Resource Facility at University of New Mexico. Mice were housed individually under conditions of constant temperature (23°C), humidity (20%), a 12 h light/dark cycle, and provided with mouse chow (standard) and water ad libitum. All experiments were conducted with AhR null mice housed 11 generations to C57Bl6N. All animal protocols were approved by the University of New Mexico Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

**Superoxide analysis in heart.** To assess in vivo levels and localization of cardiac O$_2^-$, AhR null and wild-type mice were treated with dihydroethidium (DHE, Molecular Probes, Eugene, OR). Dihydroethidium enters the cell and is oxidized primarily by O$_2^-$ to yield fluorescent products, such as ethidium (Buxser et al., 1999), which intercalates into DNA. Because ethidium fluoresces at a different wavelength (Ex = 495 nm; Em = 595 nm) than DHE (Ex = 365 nm; Em = 415 nm), ethidium fluorescence can be used to visualize localized O$_2^-$ production (Chan et al., 1998). However, the specificity of ethidium as a marker of O$_2^-$ has recently been questioned. A new molecular species has been identified, which is believed to be the specific product of DHE oxidation by O$_2^-$ (Zhao et al., 2003). This new product has a different molecular weight than ethidium, and distinct fluorescence characteristics (Ex = 480 nm; Em = 567 nm). Thus, in these experiments we used a custom-built fluorescence filter (Ex = 460–500 nm; Em = 540–585 nm) to characterize the production of the O$_2^-$–specific derived DHE product, in addition to ethidium fluorescence. Dihydroethidium was dissolved in 0.1 M phosphate-buffered saline (PBS) containing 20% dimethyl sulfoxide to a final concentration of 0.5 mM. Mice were injected ip with 1 mg/ml. Dihydroethidium–treated animals were sacrificed 6 h after treatment, and after exsanguination, the hearts were dissected, fixed in 10% neutral-buffered formalin (vol/vol) for 24 h, and sectioned at 30 μm with a vibratome. Tissue sections were viewed using excitation at 510–550 nm and emission >580 nm for ethidium detection (Olympus BH2-RFCA), and digital images were acquired (Olympus MLH202550) and analyzed with Image ProPlus. The percentage of O$_2^-$ production was quantified as the ratio of fluorescent area to total ventricular area.

**BQ-123 dosing groups.** Two-month-old AhR wild-type and null male mice were randomly assigned to either BQ-123 (American Peptide Company, Vista, CA) or vehicle (saline) treatment groups, and treated for 28 or 58 days. The BQ-123 group received 100 nmol/kg/day (dose based on personal communication with Dr. Nancy Kanagy; a dose shown to effectively reduce blood pressure in AhR null mice, Lund et al., 2005) for 28 or 58 days via osmotic mini-pumps at a flow of 0.25 μl/h (model no. 2004, Alzet, Cupertino, CA). These treatment durations were chosen in an effort to prevent the pathology (both gross tissue and molecular) associated with the onset and progression of cardiac hypertrophy observed in adult AhR null mice. The 58-day treatment period was successful in preventing the onset of cardiac hypertrophy in AhR null mice (Lund et al., 2005); thus the 28-day treatment was carried out to determine if a shorter treatment period would also be adequate. Animals were briefly anesthetized with avertin (2.5% ip at 0.015 ml/g), and mini-pumps were implanted subcutaneously. Animals were monitored daily for health status throughout the study period. No obvious side effects were noted in the BQ-123-treated animals throughout the study (no differences noted in weight, eating, drinking, or activity compared to untreated animals). One group of animals was sacrificed on day 28; plasma was collected for detection of 8-isoprostane, and hearts were collected for assessment of NAD(P)H oxidase activity. In the second group, the mini-pumps were replaced on day 29, and treatment continued until day 58. On day 58, mice were euthanized, body and heart weights were measured, and heart tissue was collected for thiobarbituric acid reactive substances (TBARS) and NAD(P)H oxidase subunit quantification.

**Plasma and tissue collection.** AhR wild-type and null mice were anesthetized with ketamine/xylazine and euthanized by exsanguination. Blood was collected in a heparinized syringe (BD Vacutainer Systems, Franklin Lakes, NJ) through cardiac puncture, and immediately centrifuged (950 × g, 10 min, 4°C) to separate plasma. Plasma was stored at −70°C for 8-isoprostane analysis, until assayed. Additionally, the heart was dissected, weighed, and frozen in liquid nitrogen. Cardiac tissue was stored at −70°C until assayed.

**8-Isoprostane analysis.** Plasma 8-isoprostane was measured in AhR null and wild-type animals using an 8-isoprostane competitive enzyme immunoassay (EIA; Caymen Chemicals, Ann Arbor, MI), following the protocol provided for determination of total (free and esterified) 8-isoprostane. All samples were purified using an 8-isoprostane affinity column (Caymen Chemicals model no. 416355) prior to analysis. Samples were run in triplicate and results were averaged. Results were calculated and quantified by a blinded participant.

**Thiobarbituric acid reactive substances (TBARS) analysis.** Cardiac tissue was resuspended by diluting 1:10 weight/volume in normal saline.
Tissue was homogenized in Potter-Elvehjem glass homogenizer, and sonicated for 15 s at 40 V. ATBARS assay kit (OXiTech, ZeptoMetrix Corp, Buffalo, NY) was used to measure TBARS levels in whole, uncentrifuged homogenates. Duplicate samples were read on a spectrophotometer (Beckman Instruments DU Series 600), and using a malondialdehyde (MDA) standard curve, and results were expressed as MDA equivalents.

**Quantification of ventricular NAD(P)H oxidase-mediated O$_2^-$ levels.** Cardiac O$_2^-$ production was assessed in ventricular samples by the lucigenin chemiluminescence method. Chemiluminescence is produced by the reaction of lucigenin with O$_2^-$ and only weakly with H$_2$O$_2$, but not with nucleoperoxidase. To prevent autooxidation of lucigenin, a with a low concentration (5 mmol/l) of lucigenin was used, as previously described (Wu et al., 2004), with the following modifications. Ventricular tissue (hearts cut transversely to include right and left ventricle) were cut in 10-μg blocks, rinsed in ice-cold PBS, and placed in cold saline on ice for 10 min. Lucigenin and PBS were added to recording tubes and incubated in the dark for 15 min. Background counts were then obtained by measuring chemiluminescence in a luminometer (Turner Designs TD-20/20 Luminometer) for 5 min (with a 1.5-min dark adjustment). Ventricular blocks were then added and measured for 5 min. To evaluate NAD(P)H oxidase activity, 100 μM NAD(P)H was then added to ventricle samples, and luminescence was measured for an additional 5 min. In some experiments, ventricle blocks were pre-incubated with 10$^{-5}$ M apocynin (4-hydroxy-3-methoxy-acetophenone), an inhibitor of superoxide production by NAD(P)H oxidases, or Tiron (4,5-dihydroxyl-1,3-benzene-disulphonic acid, 10 mmol/l), a cell-permeable nonenzymatic scavenger of O$_2^-$, for 30 min before reading. Background counts (with lucigenin) were subtracted from each value obtained from ventricular blocks. Lucigenin chemiluminescence counts were adjusted on the basis of dry weight of the ventricle blocks. Activity was expressed as relative light units (RLU)/per mg dry tissue weight/5 min.

**Gp91phox, p47phox, and p67phox mRNA analysis.** Total RNA was isolated from the left ventricle (LV) plus attached septum with Trizol (Sigma Chemical Co., St. Louis, MO). cDNA was synthesized from total RNA in a 60-μl reaction volume containing 250 ng of sample RNA, 12.5 nM of 18S reverse transcriptase (RT) primer (Table 1), 0.005 μg oligo dT, 0.0004 μl RNasin, 0.006 μl M-MLV RT enzyme, 25 mM dNTP, 12 μl 5× RT buffer, and sterile water to 60 μl volume. The mixture was heated at 42°C for 1 h and then cooled to 4°C. Real-time PCR was performed with gene-specific primers in an iCycler (Biorad, Hercules, CA). The following murine-specific primer sets were used at a concentration of 500 nM for the PCR reaction: gp91phox forward: CACCCATTCACACTGACCTCTG, gp91phox reverse: CTGCTGTTGAAGAGGACAG; p67phox forward: AGCCGGTGATATCCCCTTTCC; p67phox reverse: CTTATCAGAAAGCATTGAA; p47phox forward: CTGCTGTTGAAGAGGACAG; p47phox reverse: AGCCGGTGATATCCCCTTTCC. The reaction was initiated with denaturation at 95°C for 10 min, followed by 40 cycles at 95°C, annealing for 1 min at 60°C, and an extension at 72°C for 1 min. To confirm the presence of a single amplification product, PCR products were subjected to a melt curve analysis and were run on an agarose gel. Samples were run in triplicate and results were averaged. ΔCT (change in threshold cycle) was calculated by subtracting the CT of the 18S control gene from the CT value of the gene of interest and mean normalized gene expression was calculated as previously described (Lund et al., 2003). Results are expressed as normalized gene expression as percentage of controls.

**Statistical analysis.** Data are expressed as mean ± SEM. Statistical comparisons between two groups were performed using unpaired Student’s t-test. One-way analysis of variance (ANOVA) with the post hoc Holm-Sidak test was used for analysis of multiple groups. A value of $p < 0.05$ was considered statistically significant.

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**RESULTS**

**Elevated Superoxide in Ventricles of AhR Null Mice**

To confirm the presence and localization of ROS in the hearts of AhR null mice, DHE was used to quantify O$_2^-$ levels. Hearts of AhR null mice showed a 10-fold increase in O$_2^-$–derived fluorescence (Fig. 1A), compared to age-matched AhR wild-type mice (Fig. 1B). Fluorescence was most prominent in the left ventricle, and it was also present in the right ventricle (data not shown), and was detectable inside the nucleus of cardiac myocytes (Fig. 1D). No fluorescence was observed in AhR null heart sections that were not treated with DHE (Fig. 1C).

**Effects of BQ-123 on Heart Weights in AhR Null Mice**

To study the role of ET-1–ET$_A$–mediated effects on cardiac hypertrophy in AhR null mice, both AhR null and wild-type mice were chronically treated with a specific ET$_A$ receptor antagonist, BQ-123 (Ihara et al., 1992), for 28 days. BQ-123 was chosen for the experiments reported here because the ET$_A$ receptor has been shown to primarily mediate hypertrophy signaling pathways in the heart (Schunkert et al., 1999; Shubeita et al., 1990); as well as ROS induction in cardiac myocytes (Xu et al., 2004). BQ-123 significantly decreased both heart weight and the heart-to-body weight (HW/BW) ratio in AhR null mice compared to untreated AhR null mice, and it reduced values to AhR wild-type levels (Table 1).

**TABLE 1** Gross tissue weights in AhR null and wildtype mice treated with saline or BQ-123 for 28 days

<table>
<thead>
<tr>
<th></th>
<th>Heart weight (g)$^a$</th>
<th>Body weight (g)</th>
<th>HW/BW ratio (×100 g)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR +/+</td>
<td>0.144 ± 0.006</td>
<td>30.4 ± 0.5</td>
<td>0.476 ± 0.004</td>
</tr>
<tr>
<td>AhR +/+ Rx</td>
<td>0.143 ± 0.007</td>
<td>29.9 ± 0.5</td>
<td>0.479 ± 0.004</td>
</tr>
<tr>
<td>AhR −/−</td>
<td>0.171 ± 0.005$^*$</td>
<td>32.5 ± 1.5</td>
<td>0.530 ± 0.014$^*$</td>
</tr>
<tr>
<td>AhR −/− Rx</td>
<td>0.150 ± 0.006$^+$</td>
<td>30.6 ± 1.0</td>
<td>0.490 ± 0.005$^+$</td>
</tr>
</tbody>
</table>

Values are means ± SE. AhR +/+, wildtype; AhR −/−, null. Rx, BQ-123-treated group. $^a$p < 0.050 compared to AhR +/++; $^b$p < 0.050 compared to untreated AhR −/−. All four groups reported $n = 8$. $^*$ANOVA $p < 0.001$; $^+$ANOVA $p < 0.1$.
oxygen radicals. At least one of the isoprostanes, 8-isoprostane (8-epi PGF2), has been shown to have biological activity and is used as a marker of antioxidant deficiency and oxidative stress (Morrow et al., 1995). AhR null mice exhibit significantly increased levels of plasma 8-isoprostane, compared to AhR wild-type mice (Fig. 2). BQ-123-treatment significantly reduced plasma 8-isoprostane levels in AhR null mice, compared to untreated AhR null mice, to values that were not significantly different from controls at \( p < 0.05 \) (Fig. 2). There was no difference in plasma 8-isoprostane between BQ-123–treated and untreated wild-type mice.

**BQ-123 Attenuates Cardiac TBARS in AhR Null Mice**

Having found that ET-1 blockade reduced plasma 8-isoprostane levels, we next investigated the effects of BQ-123 on ROS production in the heart by measuring thiobarbituric acid reactive substances (TBARS), which assess lipid peroxidation, an indicator of oxidative stress. Cardiac TBARS levels were significantly decreased in BQ-123–treated AhR null mice, compared to untreated AhR null mice, but they remained elevated compared to AhR wild type (Fig. 3). No differences were noted between cardiac TBARS in untreated and BQ-123–treated wild-type mice. These findings suggest that ROS levels in hearts of AhR null mice are largely mediated through ET-1–ET\(_ A\) pathways.

**BQ-123 Reduces NAD(P)H-Dependent ROS Generation in the Hearts of AhR Null Mice**

Lucigenin chemiluminescence was used to assess NAD(P)H oxidase activity in ventricles from AhR null and wild-type
ET-1-MEDIATED ROS IN HEARTS OF AHR NULL MICE

FIG. 2. Plasma 8-isoprostane in AHR null and AHR wild type mice after 28-day saline or BQ-123. BQ-123 reduced plasma 8-isoprostane in AHR null mice compared to AHR null mice and reduced it to levels that were not significantly different from control at \( p < 0.05 \). AHR \( +/- \), AHR wild type; AHR \( +/- \) Rx, BQ-123-treated AHR wild type; AHR \( +/- \) Rx, AHR null; AHR \( +/- \) Rx, BQ-123-treated AHR null. * \( p < 0.05 \) compared to AHR \( +/- \) mice, \( \bar{p} < 0.05 \) significantly different compared to AHR null mice. AHR \( +/- \) (n = 6), AHR \( +/- \) Rx (n = 6), AHR \( +/- \) (n = 6); AHR \( +/- \) Rx (n = 6), \( \bar{p} = 0.053 \) compared to AHR \( +/- \) Rx; \( \bar{p} = 0.063 \) compared to AHR \( +/- \).

mice. In the ventricles, lucigenin chemiluminescence was induced nearly 2-fold in AHR null mice, compared to the AHR wild type (Fig. 4). BQ-123-treatment significantly reduced lucigenin chemiluminescence in the ventricles of AHR null mice to wild-type levels, whereas no significant change in \( \text{O}_2^- \) production was measured between untreated and BQ-123-treated wild-type mice. Sources of NAD(P)H-dependent ROS production were examined in hearts from AHR null mice via use of the specific inhibitor apocynin, which inhibits superoxide formation by preventing the assembly of the superoxide-generating enzyme NADPH oxidase, and Tiron, a non-enzymatic cell-permeable \( \text{O}_2^- \) scavenger, in the lucigenin assay. Reactive oxygen species production in the hearts of AHR null mice was reduced to wild-type levels by both Tiron and apocynin pretreatment (Fig. 4), suggesting that the source of cardiac ROS is primarily NADPH oxidase-derived \( \text{O}_2^- \).

Effects of BQ-123 on NAD(P)H Oxidase Subunit Expression in the Hearts of AHR Null Mice

All NAD(P)H oxidase subunit, gp91 \( \text{phox} \), p47 \( \text{phox} \), and p67 \( \text{phox} \), mRNAs were detected in the hearts of AHR null and wild-type mice. In AHR null mice, expression of gp91 \( \text{phox} \) mRNA was 4.5-fold higher than in AHR wild type (Fig. 5). Furthermore, regulatory subunits p47 \( \text{phox} \) and p67 \( \text{phox} \) were also found to be upregulated in hearts of AHR null mice. 4.7- and 4.3-fold, respectively, compared to the wild type, indicating increased expression of NAD(P)H oxidase. BQ-123 treatment significantly attenuated cardiac mRNA expression of gp91 \( \text{phox} \), p47 \( \text{phox} \), and p67 \( \text{phox} \) to wild-type levels. No statistical difference was detected between expression of gp91 \( \text{phox} \), p47 \( \text{phox} \) or p67 \( \text{phox} \) in untreated and BQ-123–treated AHR wild-type mice. Such findings confirm that NAD(P)H oxidase expression is upregulated in the hearts of AHR null mice.

DISCUSSION

The present study demonstrates that cardiac hypertrophy in AHR null mice is associated with elevated ROS, specifically \( \text{O}_2^- \). Furthermore, the induction of ROS in the hearts of AHR null mice is mediated, in part, through ET-1 activation of the
ETα receptor, because chronic ETα receptor blockade reduces ROS levels in the heart nearly to wild-type levels. Thus, AhR null mice exhibit cardiovascular disease that is ET-1–ETα-mediated and is associated with oxidative stress. In this regard, AhR null mice resemble other rodent models of ET-1–dependent cardiovascular disease, such as rats chronically infused with Ang II, DOCA-salt rats, and Dahl salt-sensitive rats (Nakagami et al., 2003; Pollock, 2005). However, AhR null mice represent a particularly novel model of ET-1–ETα-mediated cardiac hypertrophy and associated elevations in ROS, because these pathological and physiological alterations occur in the absence of any additional external stimuli or manipulation, such as a high-salt diet or Ang II infusion.

The development of cardiac hypertrophy in AhR null mice is well documented (Fernandez-Salgueiro et al., 1997; Lund et al., 2003; Thackaberry et al., 2002; Vasquez et al., 2003), and a previous study has shown that ET-1 is the primary mediator of the cardiac hypertrophy (Lund et al., 2005). Endothelin-1 is associated with generation of ROS in cardiomyocytes (Hirotani et al., 2002), which are believed to mediate ET-1–mediated cardiac hypertrophy. This premise has been confirmed by study findings which show that ETα receptor antagonism attenuates cardiac hypertrophy in experimental models (Ehmke et al., 1999; Ito et al., 1994). Thus, 2-month-old AhR null mice were treated for 28 days with the ETα receptor antagonist BQ-123. In agreement with previously cited studies (Ehmke et al., 1999; Ito et al., 1994), we found that ETα blockade significantly decreases heart weight and the heart weight-to-body weight ratio in AhR null mice.

To characterize the ability of ETα blockade to reduce ROS associated with cardiac hypertrophy in AhR null mice, we used two independent measures of ROS generation: cardiac TBARS, a nonspecific assessment of ROS production, and plasma 8-isoprostane, a systemic product that forms as the result of oxidation of phospholipids by ROS (Roberts and Morrow, 2000). Cardiac TBARS are significantly elevated in AhR null mice, and the increase over that measured in AhR wild-type mice is similar to inductions previously reported in both animal and human models of cardiac hypertrophy (Luo et al., 2002; Motoyama et al., 2001). Additionally, plasma 8-isoprostane levels are also significantly increased in AhR null mice, compared to AhR wild-type mice; and similar inductions have also been reported in other models of cardiovascular pathology, such as hypertension (Patterson et al., 2005). ETα blockade significantly reduces both cardiac TBARS and plasma 8-isoprostane in AhR null mice; however, levels of both of these indicators of ROS remain elevated compared to AhR wild-type mice, suggesting that induction of ROS in AhR null mice is only partially mediated by ET-1 activation of ETα receptors. One possible explanation for the inability of ETα blockade to completely reduce TBARS and 8-isoprostane levels is that plasma Ang II remains significantly elevated in AhR null mice, but not in wild-type mice, after chronic ETα antagonist therapy (Lund et al., 2005). Angiotensin II has been shown to increase cardiac ROS production (Nakagami et al., 2003) and thus, elevated levels of Ang II in AhR null mice may represent an additional contributor to the production of cardiac and plasma ROS. Future studies involving both ETα and Ang II receptor antagonism will help to define the roles of these two vasoactive peptides in ROS production in AhR null mice.

In an effort to determine the source of elevated ROS observed in the myocardium in AhR null mice, we assessed NAD(P)H oxidase activity. The NAD(P)H oxidases of the cardiovascular system are membrane-associated enzymes that catalyze the 1-electron reduction of oxygen using NADH or NAD(P)H as the electron donor. In vitro studies suggest that...
ET-1 mediates NAD(P)H oxidases in several cell types (Duerrschmidt et al., 2000; Fei et al., 2000), including cardiac myocytes (Tanka et al., 2001). Our results show that NAD(P)H oxidase-generated ROS, as measured by lucigenin chemiluminescence, is increased in hearts of AhR null mice. Furthermore, the origin of ROS appears to be mediated by NAD(P)H oxidase, because pretreatment with apocynin, an inhibitor of superoxide production by NAD(P)H oxidases, suppresses the increased lucigenin activity to control levels. Finally, this induction of NAD(P)H oxidase appears to be mediated through ET-1–ETα receptor activation because BQ-123 significantly reduces NAD(P)H oxidase activity. These data indicate that cardiac hypertrophy in AhR null mice is associated with increased NAD(P)H oxidase induction, resulting in elevation of cardiac ROS levels.

These findings are further confirmed through analysis of expression of cardiac NAD(P)H oxidase components, gp91phox, p67phox, and p47phox. The gp91phox, along with the p22phox, are integral membrane proteins; whereas the p67phox and p47phox subunits are located in the cytosol. Upon activation, p47phox is phosphorylated and translocated with p67phox to the gp91phox–p22phox core oxidase, resulting in O2•− production. Expression of each of these subunits has been confirmed in cardiac myocytes (Xiao et al., 2002), and NAD(P)H oxidase can be regulated over the long term by upregulation of transcription of the oxidase subunits (Lességue and Clempus, 2003; Touyz et al., 2002). Real-time PCR analysis of mRNA expression of these NAD(P)H oxidase subunits shows that all are significantly elevated in AhR null mice, when compared to the wild type. BQ-123 treatment results in a decrease in expression of gp91phox, p67phox, and p47phox in AhR null mice. Such findings suggest that NADPH oxidase expression is regulated through an ET-1–ETα-dependent mechanism in hearts of AhR null mice. Whereas ET-1 has previously been shown to increase NAD(P)H oxidase activity in endothelial cells (Duerrschmidt et al., 2000), and to slightly increase activity in vascular smooth muscle cells (Touyz et al., 2004), to our knowledge these findings are the first to report that ETα blockade results in decreased expression of NAD(P)H oxidase subunits (gp91phox, p67phox, and p47phox), suggesting that ET-1 mediates NAD(P)H oxidase activity in cardiac tissue. These findings also suggest that any Ang-II derived ROS present in hearts of AhR null mice are not NAD(P)H oxidase derived, because NAD(P)H oxidase activity is normalized through BQ-123 treatment.

Previous studies have shown that both Ang II and ET-1 contribute to cardiac hypertrophy observed in AhR null mice (Lund et al., 2003, 2005) and both of these cardiac mitogenic peptides are believed to mediate their growth-promoting effects on the heart by increased production of ROS (Tanka et al., 2001). It has been proposed that ROS may mediate the cardiac hypertrophic response by acting as a regulator of gene expression (Kunsch and Medford, 1999), either through direct activation of G proteins (Chiloeches et al., 1999; Nishida et al., 2002) or by altering activity of other growth-promoting signaling pathways, such as MAPKs (Kyaw et al., 2002). Thus, it is tempting to speculate that induction of ROS by ET-1 and/or Ang II mediates the development and progression of cardiac hypertrophy in AhR null mice. Although the present study demonstrates that ET-1 increases cardiac ROS via ETα receptors in AhR null mice, more experiments are needed to delineate the causative role of ROS in the development and progression of the cardiac hypertrophy. The results reported here suggest a role for O2•− in the progression of cardiac hypertrophy in AhR null mice; however, we have not yet determined whether other O2•− derived ROS, such as hydrogen peroxide (H2O2), may also contribute to the pathology of the myocardium.

In conclusion, we have shown that AhR null mice exhibit cardiac hypertrophy, which is associated with increased production of cardiac ROS. Furthermore, chronic treatment with an ETα receptor antagonist, BQ-123, results in a significant reduction of cardiac hypertrophy and NADPH oxidase–derived O2•− production in hearts of AhR null mice. Such results suggest that ET-1 mediates the increase in cardiac NAD(P)H oxidase–induced O2•− production in AhR null mice through an ETα receptor-signaling pathway. The mechanisms by which ROS may contribute to the progression of cardiac hypertrophy in AhR null mice remains to be determined, and it can likely be clarified through studies involving antioxidant therapy. Given that recent studies have identified endogenous ligands of the AhR (Song et al., 2002; reviewed in Denison and Nagy, 2003), future studies that further elucidate the role of AhR in cardiovascular physiology may provide signaling pathways that serve as mediators of the induction or progression of cardiac hypertrophy.

**ACKNOWLEDGMENTS**

This study was supported by National Institute of Environmental Health Sciences (NIEHS) Center Grant P30ES12102 and ES10433 to M.K.W. and a predoctoral fellowship from the Environmental Protection Agency, EPA STAR – U91621501, to A.K.L.

**REFERENCES**


Chiloeches, A., Paterson, H. F., Marais, R. M., Clerk, A., Marshall, C. J., and Sugden, P. H. (1999). Regulation of Ras.GTP loading and Ras-Raf signaling pathways, such as MAPKs (Kyaw et al., 2002).


