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The Aryl-hydrocarbon receptor does not require the p23 co-chaperone for ligand binding and target gene expression in vivo

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Abstract

The Aryl-hydrocarbon receptor (*Ahr*) is a ligand-activated transcription factor that mediates most of the toxic affects of 2,3,7,8-tetrachlorodibenzo-(*p*)-dioxin (TCDD) and other xenobiotic compounds. The AHR cytoplasmic complex consists of two molecules of HSP90 and at least one molecule of Hepatitis B Virus-X associated protein 2 and the co-chaperone p23. With the use of in vitro model systems, p23 has been shown previously to be important to maintaining the efficient ligand binding and subsequent downstream inducibility of the AHR. In this study we attempted to identify the role p23 plays in AHR signaling in vivo using a *p23* null mouse. Ligand binding assays and western blot analysis revealed that p23 was not required for AHR protein stability and competent ligand binding in liver. Real-time RT-PCR analysis conducted on *p23* null, heterozygous and homozygous mice suggested that p23 is dispensable for stable AHR protein levels, or efficient TCDD-mediated AHR activation of *Cyp1a1* and *Cyp1a2*.

Keywords

p23; HSP90; AHR; TCDD

1. Introduction

The Aryl-hydrocarbon receptor (AHR) is a component of a ligand-activated heterodimeric transcription factor complex that includes the ARNT (Aryl-hydrocarbon receptor nuclear translocator) protein (reviewed in (Beischlag et al., 2008). AHR binds a number of ligands that include 2,3,7,8-tetrachlorodibenzo-(*p*)-dioxin (TCDD, dioxin) and numerous other important xenobiotic compounds. Mice engineered to lack *Ahr* expression have been important in linking both the toxicity of ligands and endogenous biological actions associated with AHR function (Fernandez-Salguero et al., 1996; Lahvis et al., 2005; Rodriguez-Sosa et al., 2005). In addition to its direct effects on gene expression, AHR also functions by modifying the activity of other transcription factors through a variety of

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Conflict of interest
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mechanisms that includes coupling to proteosomal degradation pathways (Ohtake et al., 2007).

AHR function is regulated in part by the HSP90 family of ATP-dependent chaperone proteins (for a current and comprehensive review, see http://www.picard.ch/downloads/Hsp90facts.pdf). HSP90–AHR complexes can be isolated from cells and HSP90 inhibitors, such as geldanamycin, block AHR signaling (Meyer et al., 2000; Perdew and Bradfield, 1996). HSP90 physically interacts with both the basic helix–loop–helix domain and the PAS B/ligand binding domain of AHR (Perdew and Bradfield, 1996). The interaction with HSP90 is thought to shape AHR's ligand binding domain into a state that is competent for ligand binding, and it also serves to negatively regulate AHR until ligand binding occurs. Genetic and pharmacological studies conducted in yeast suggest HSP90 isotype specific regulation for AHR, with the HSC82 protein (homolog of human HSP90β) of yeast providing the best support for AHR signaling (Cox and Miller, 2003; Yao et al., 2004). Several distinct co-chaperone proteins confer substrate protein specificity to the HSP90 proteins and, in the case of AHR, the two HSP90 co-chaperones most frequently studied are Hepatitis B Virus-X associated protein 2 (XAP2) and p23. XAP2 (also called ARA9 or AIP) is a member of the immunophilin class of proteins and its contact sites with HSP90 and AHR are mediated through multiple tetratricopeptide repeat (TPR) motifs (Meyer and Perdew, 1999; Meyer et al., 1998). The XAP2–HSP90 complex protects AHR from proteolytic degradation through a mechanism that involves the E3 ubiquitin ligase action of CHIP and it also serves as a negative regulatory complex in the absence of ligand activation (Lees et al., 2003; Morales and Perdew, 2007). In a synthetic yeast model system, low levels of XAP2 expression enhanced human AHR signaling, whereas high level expression of XAP2 blocked AHR signaling, which is consistent with both stabilizing and negative regulatory roles reported in other systems (Miller, 2002). XAP2 is also found in HSP90-peroxisome proliferator activator receptor (PPAR) complexes, and mice lacking XAP2 have an embryonic lethal phenotype with cardiac defects that are distinct from the defects in *Ahr*-null mice (Hollingshead et al., 2006; Lin et al., 2007).

The p23 protein (also called cytosolic prostaglandin E2 synthase; cPGES, and telomere end binding protein; TEBP) was first described as a HSP90 co-chaperone found in complexes with some steroid hormone receptors (Johnson et al., 1994; Smith and Toft, 1992).

Numerous biological activities have been ascribed to p23, and it has distinct functions both with and in absence of HSP90 (for a current comprehensive review, see http://www.picard.ch/downloads/p23facts.pdf). The p23 protein has intrinsic chaperone ability in that it prevents aggregation of proteins, plays a role in telomerase processivity, and acts as a glutathione-dependent prostaglandin E2 synthase that displays enhanced activity when HSP90 is present (Freeman et al., 1996; Tanioka et al., 2003; Toogun et al., 2007). A key aspect of p23 function is that it preferentially associates with the ATP-bound form of HSP90 to prolong the half-life of the nucleotide associated state by slowing hydrolysis (Sullivan et al., 1997). The p23 protein is found in HSP90 complexes containing glucocorticoid and progesterone receptors (GR and PR respectively) when isolated in the presence of molybdate or non-hydrolyzable ATP analogs. In biochemical reconstitution studies, the ultimate addition of p23 shifts GR into a high affinity state that binds biologically relevant concentrations of ligands (Pratt et al., 2006). p23 does not appear to directly contact steroid hormone receptors, but instead mediates effects indirectly through its association with HSP90. In addition to its role in capacitating some receptors for ligand binding, p23 complexes with HSP90 and other co-chaperones provide for rapid cycling or removal of specific steroid hormone receptors on DNA (Freeman and Yamamoto, 2002; Meijsing et al., 2007; Stavreva et al., 2004).

AHR is similar to the GR in its dependency on HSP90 interactions. Transient transfections using a mutated form of *Ahr* suggest that XAP2 and p23 binding is mutually exclusive within AHR–HSP90 complexes, and that XAP2 may not be required for AHR signaling (Hollingshead et al., 2004). p23-associated HSP90 complexes were reported to regulate AHR via a pendulin (importin) dependent process that in turn mediated transport into the nuclear compartment (Kazlauskas et al., 1999). In contrast, Shetty et al. (2003) reported that purified p23 was needed to form AHR–ARNT–DNA complexes in vitro through a HSP90 dependent process. Using a yeast genetic model system, Cox and Miller (2002, 2004) showed that p23 was required for the potency and efficacy of ligand-induced AHR signaling, and that p23 over-expression restored human AHR signaling to yeast with a specific HSP90 mutation that originally compromised signaling. Thus, there is considerable suggestive evidence of a role for p23 in AHR signaling.

Mice lacking *p23* display lethality at birth, indicating the essential nature of this protein (Grad et al., 2006; Lovgren et al., 2007; Nakatani et al., 2007). Subsequent studies using late stage *p23* null embryos revealed defects in lung development that were similar to defects described for mice lacking GR or corticotropin releasing hormone. Fibroblastic cell lines derived from *p23* null embryos displayed defects in GR ligand binding and signaling (Grad et al., 2006). Thus, the absence of p23 likely impairs HSP90-regulated steroid hormone receptor signaling processes.

The objective of the studies presented here was to test whether a role for p23 in AHR signaling could be demonstrated. We performed these studies with hepatic tissues of mice that expressed different levels of p23. To circumvent the problem of perinatal lethality in *p23* null animals, we utilized fetal hepatic tissues harvested a day before scheduled parturition. AHR, ARNT, and XAP2 expression levels, expression of endogenous *Cyp1a1* and *Cyp1a2* gene expression targets of AHR signaling and photoaffinity ligand binding assays in the context of different genetic backgrounds were used to determine the impact of *p23* on AHR function in vivo.

2. Materials and methods

2.1. Mouse genotyping, handling and AHR ligand treatment

Protocols for maintaining and treating mice were approved by Tulane University's Institutional Animal Care and Use Committee. Murine genotypes were determined by PCR analysis and by immunoblot analysis of p23 protein expression as described previously (Grad et al., 2006).

2.2. Toxicity study

Adult female mice of *p23* heterozygous status and their homozygous littermates were treated with TCDD (10 μg/kg, i.p.) in olive oil or vehicle alone and sacrificed 8 days later. The mice were approximately 6 months old and at backcross six in the progression toward a full C57Bl/6 genetic background. Livers from these mice were assessed for weight and histological changes and provided the sources of mRNA and protein for subsequent analyses. Sections of liver were fixed and stained with hematoxylin and eosin and examined by a pathologist. Differences in liver weight:body weight ratios were compared for significance using one-way ANOVA and Tukey's post test.

2.3. Acute embryonic AHR activation

For studies in late stage fetal mice, we bred *p23* heterozygous mouse pairs that were backcrossed nine generations or higher toward a C57Bl/6 genetic background. Females were examined each morning, and those with copulatory plugs were considered to be at

gestational day 0.5. The near-term (gestational day 18.5) fetal mice were harvested; livers were isolated, and analyzed for ligand binding, mRNA induction and protein expression experiments. For the *in utero* transcriptional activation experiments, pregnant females at gestational day 18.5 were injected (i.p.) with 20 mg/kg β-naphthoflavone (β-NF) in olive oil. Six hours post-injection the fetal and maternal livers were harvested, stored in liquid nitrogen, and then and total mRNA and proteins were subsequently isolated as described below.

2.4. Real-time RT-PCR

Total mRNA was isolated from mouse liver sections using Tri-Reagent (Sigma). One microgram of each mRNA sample was used to synthesize cDNA using ABI cDNA archive synthesis kit (ABI) as described by the manufacturer. mRNA expression was quantified using real-time RT-PCR (Biorad) with the following conditions; 95.0 °C for 3 min, 95.0 °C for 20 s 60.0 °C for 20 s and 72.0 °C for 45 s for ×40 cycles. Hepatic mRNA expression were quantified using the following genes and their respective primers; C*yp1a1*, C*yp1a2*, *β-actin*, and ribosomal protein L13 (see Supplementary Table S1 for sequences).

2.5. Immunoblotting

To generate cytosol for ligand binding experiments, mouse livers were homogenized in MENG buffer (25 mM MOPS, 2 mM EDTA, 0.02% NaN_3, 10% glycerol pH 7.4) containing 20 mM sodium molybdate and protease inhibitors (Sigma) and centrifuged at 100,000 × *g* for 1 h. Total hepatic protein were isolated using Tri-Reagent (Sigma). Twenty micrograms of total protein/lane was resolved using 8% acrylamide-TSDS-PAGE, transferred to polyvinylidene diflouride (PVDF) membrane and probed using antibodies for p23 (JJ3 a gift from David Toft, Mayo Clinic), XAP2 (Novus Biologicals), AHR (RPT1 antibody, Affinity Bioreagents) β-actin (Santa Cruz Biotechnology) along with the cognate iodinated goat-anti-mouse or goat-anti-rabbit secondary antibodies (Amersham Biosciences). The immunoreactive proteins were then visualized using autoradiography.

2.6. Photoaffinity ligand synthesis and binding assays

The photoaffinity ligand 2-azido-3-[^{125}]iodo-7,8-dibromodibenzo-*p*-dioxin (PAL) was synthesized as described previously (Poland et al., 1986; Ramadoss and Perdew, 2004). All binding experiments were conducted in the dark until UV-mediated activation of the photoaffinity ligand was completed. A saturating amount of the photoaffinity ligand (0.21 pmol i.e.: 8×10^5 cpm per tube) was added to 150 μg total protein of mouse liver cytosol and incubated at room temperature for 20 min. The photoligand was activated at 8 cm with 402 nm UV light. One percent dextran coated charcoal was added to the photolyzed samples, which were then centrifuged at 3000 × *g* for 10 min to remove free ligand. For PAL displacement experiments, saturating amounts of PAL and a 200-fold molar excess of dimethyl-indolo[3,2-*b*]carbazole (MICZ) were added simultaneously to the binding reaction. Labeled samples were resolved using 8% acrylamide-TSDS-PAGE, transferred to PVDF membrane and visualized using autoradiography. Ligand bound AHR bands were excised and counted using a γ-counter (Amersham Biosciences).

2.7. Statistical analysis

Mouse liver weight data was analyzed using one-way ANOVA and Tukey's post test analysis. Real-time RT-PCR data was analyzed using two-way ANOVA and Bonferroni post tests. All statistical calculations were performed using GraphPad Prism software (GraphPad Software Inc. San Diego, CA). Calculated *p* values <0.05 were considered statistically significant.

3. Results

3.1. Homozygous p23 expression is not necessary for TCDD-induced toxicity

Hepatomegaly is a hallmark of TCDD-induced liver toxicity requiring AHR expression in hepatocytes (Walisser et al., 2005). Therefore we investigated whether p23 expression played a significant role in AHR-mediated toxicity. Treatment of adult *p23* homozygous and heterozygous female mice with TCDD (10 μg/kg) resulted in no overtly toxic symptoms and no changes in weight at the time of harvest on day 8 post-exposure (Fig. 1). The TCDD treated animals displayed mild hepatic inflammation and there were no discernable differences between samples from p23 homozygous and heterozygous mice (data not shown). A small but statistically significant increase in liver weight:body weight ratio was observed in the *p23* homozygous mice, but no other treatment effects that comprise overt toxicity and hepatomegaly were observed (Fig. 1). These findings highlighted the fact that a high level of p23 expression is not necessary for AHR-mediated TCDD-induced toxicity.

3.2. Heterozygous p23 expression is sufficient for efficient induction of TCDD-responsive genes and AHR protein stability

p23 heterozygous and homozygous female mice treated with TCDD or vehicle control displayed no significant differences in *Cyp1a1* or *Cyp1a2* mRNA induction in response to TCDD treatment (Fig. 2a). Western blot analysis of whole liver cytosol generated from *p23* heterozygous and homozygous mice detected no significant differences in AHR or XAP2 expression levels; despite reduced expression of p23 in *p23* heterozygous mice, compared to *p23* homozygous mice (Fig. 2b).

3.3. p23 expression is not necessary for stable AHR protein expression, or competent AHR ligand binding

AHR present in protein extracts, isolated from either *p23* homozygous, heterozygous or null mouse embryonic livers, was able to competently bind saturating amounts of PAL. There was no significant difference in the amount of PAL bound to the AHR from each mouse genotype (Fig. 3a and b). AHR and XAP2 levels did not significantly differ in p23 null embryos compared to p23 homozygous mouse embryos (Fig. 3a lower panels).

3.4. p23 expression is not necessary for AHR activation of gene expression

To investigate if p23 expression was important to AHR activation of TCDD-responsive genes, β-naphthoflavone (β-NF) was used to induce hepatic *Cyp1a1* mRNA expression in both $p23^{-/-}$ and $p23^{+/-}$ murine embryos and maternal tissue. Pregnant $p23^{+/-}$ dams at gestational day 18.5 were injected (i.p.) with 20 mg/kg β-NF in olive oil, embryonic and maternal livers were harvested, and total RNA was isolated. Levels of *Cyp1a1* mRNA expression were measured using real-time RT-PCR. *In utero* transcriptional activation of the AHR in *p23* null and *p23* heterozygous embryonic liver revealed no differences in *Cyp1a1* expression in response to β-NF treatment (Fig. 4a and b). Thus, p23 expression had no influence on the modulation of *Cyp1a1* expression by the AHR.

4. Discussion

Previous studies have highlighted the importance of p23 protein, acting in concordance with HSP90, in the regulation of a number of diverse receptor signaling pathways. p23 has also been shown to be important in maintaining GR in a high affinity ligand binding state (Pratt et al., 2006). Conversely, in vitro analysis has shown that p23, along with HSP90, is important to maintaining the AHR protein in a ligand inducible conformation (Cox and Miller, 2002, 2004; Hollingshead et al., 2004; Shetty et al., 2003). Interestingly, our investigations into the effect of p23 expression on AHR ligand binding and gene regulation

suggest that p23, although shown to be a component of the stable AHR cytoplasmic multiprotein complex, may not be indispensable for efficient AHR stability and gene regulation. As a result, these discoveries indicate that there may be functional dissimilarity between AHR and GR interaction with p23 proteins, which suggests, more specifically, that p23 may actually be less important to AHR signaling.

It is important to note that understanding the precise role p23 plays in AHR-mediated gene regulation may only be obtainable through complete abrogation of p23 expression in adult knockout mice, which is not possible due to *p23* null mice embryonic lethality. This is true even after considering our findings that reduced p23 expression in heterozygous mice had no significant effect on AHR activity. Contrastingly, a statistically significant increase in liver weight:body weight ratio was observed in the *p23* homozygous but not *p23* heterozygous mice. The small but significant increase in liver size observed was surprising considering the intermediate dose of TCDD used. In addition, no other effects of TCDD-induced hepatomegaly were observed in these *p23* homozygous mice. This suggested that the observed increased liver weight:body weight ratio in *p23* homozygous mice although statistically significant may be biologically inconsequential. However, we have shown through ligand binding experiments that p23 expression may not be necessary for competent AHR ligand binding, as proposed in previous investigations. Although the implications of the negative results we obtained in this study can only be cautiously extrapolated. For example, it is possible that hepatic cells from *p23* heterozygous or null animals have adapted to reduced p23 levels by altered regulation of existing or new AHR chaperoning activities or employed other mechanisms that remain to be discovered.

A BLAST search with the murine p23 protein sequence against the murine proteome revealed the presence of additional proteins with p23 sequence similarity (C. Miller, unpublished data). Also, *p23* and its homologue *tsp23*, which are expressed in a complementary tissue-specific manner, have been shown to differentially alter intracellular receptor signaling and thus may be involved in tissue-specific ligand responsiveness (Freeman et al., 2000). Thus, it is possible that there may be additional p23-like proteins that can functionally compensate in AHR regulation when p23 is absent or that may actually play a significant role in AHR signaling in hepatic tissue. If this is the case, then the role of p23-like proteins in AHR signaling could be elucidated through disrupting expression of multiple genes such as through targeting with siRNA. Nonetheless the apparent dispensability of p23 to AHR function highlighted through our investigations serves to showcase a potential redundant role of p23 in AHR function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.toxlet.2009.05.002.

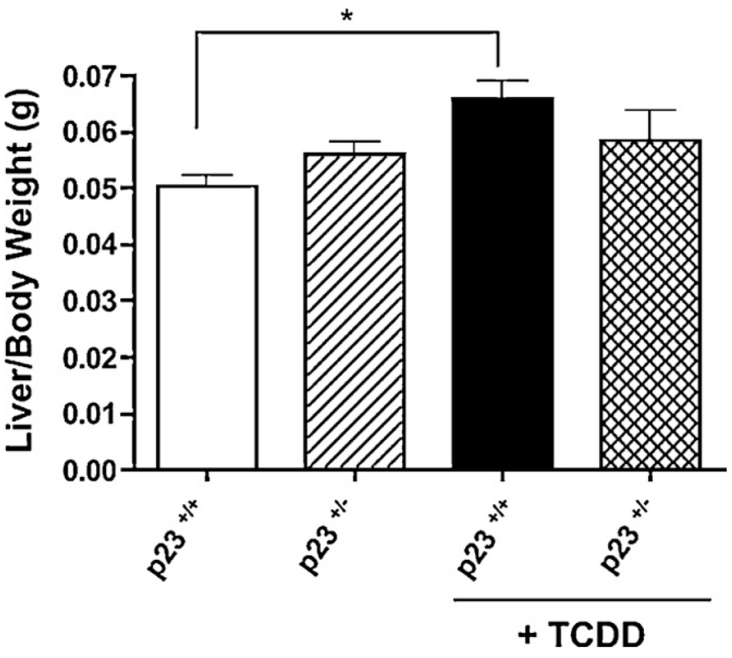

Fig. 1.

Treatment of p23 heterozygous and homozygous mice with TCDD results in mild toxicity. Adult female mice of *p23* heterozygous status and their homozygous littermates were treated with TCDD (10 μg/kg, i.p.) in olive oil or vehicle alone and sacrificed 8 days later. Livers from these mice were assessed for weight and histological changes and provided the sources of RNA and protein for subsequent analyses. Differences in liver weight:body weight ratios were compared for significance using one-way ANOVA and Tukey's post test.

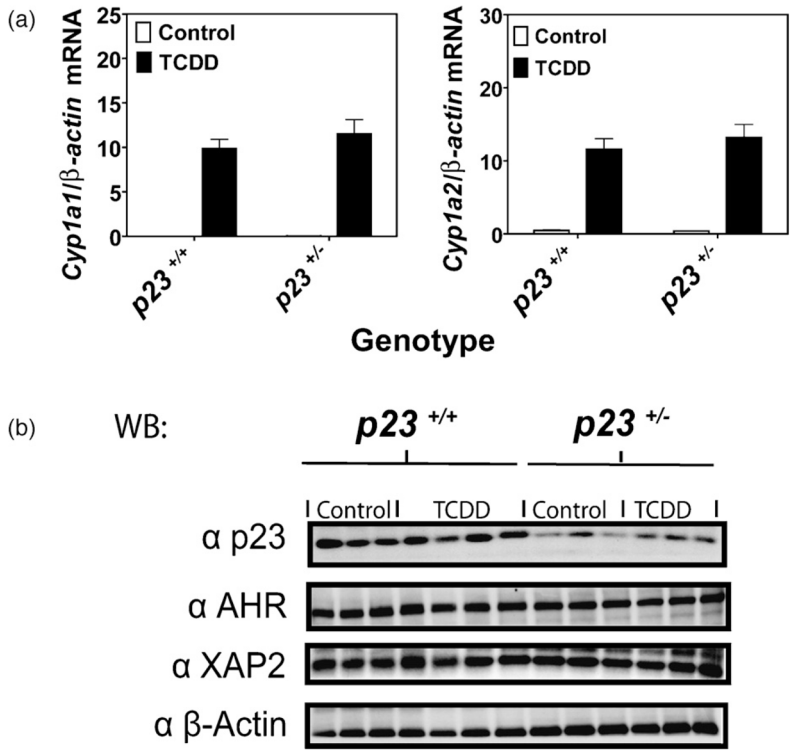

Fig. 2.

Reduced expression of p23 does not affect *AHR* expression levels or induced transcripts of *Cyp1a1* and *Cyp1a2* in mice exposed to TCDD. (a) $p23^{+/+}$ and $p23^{+/-}$ were treated with 10 μg/kg TCDD or olive oil control. Eight days post-exposure the mice were sacrificed and assessed for hepatic *Cyp1a1* and *Cyp1a2* mRNA expression using real-time RT-PCR. (b) Hepatic protein extracts from *p23* heterozygous mice display reduced p23 expression, which has no significant influence on AHR, or XAP2 protein levels. Total hepatic protein was isolated using Tri-Reagent (Sigma). Protein was resolved using 8% acrylamide-tricine SDS-PAGE, transferred to polyvinylidene diflouride (PVDF) membrane and probed using antibodies for p23, AHR and β-actin.

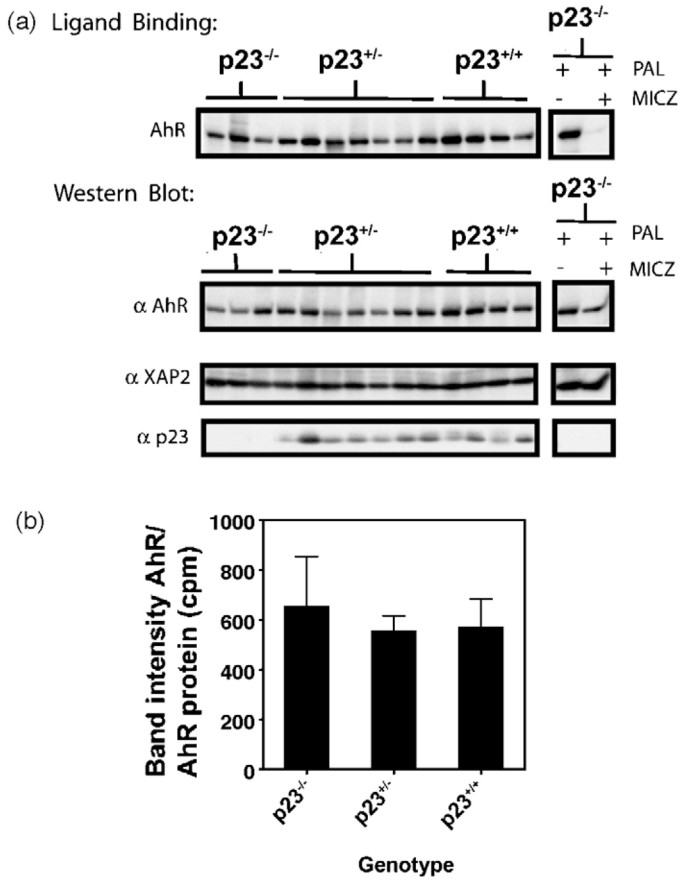

Fig. 3.

p23 expression is not required for efficient AHR ligand binding. (a) Liver cytosol from *p23* null and homozygous embryos (gestational day 18.5) display equivalent binding affinities for the PAL. A saturating amount of PAL was added to each tube containing liver cytosol and photo-linked to AHR. 200-fold molar excess of the unlabeled AHR ligand 5,11-dimethylindolo[3,2 *b*]carbazole (MICZ), was added with saturating amounts of PAL in order to, verify reaction specificity (upper right sub panel). Labeled samples were resolved using acrylamide-tricine SDS-PAGE transferred to PVDF membrane and visualized using autoradiography. The presence of XAP2, p23 and AHR was assessed using western blot analysis. (b) AHR bands resolved in panel A were excised and the respective radioactivity levels were compared using a γ-counter and plotted.

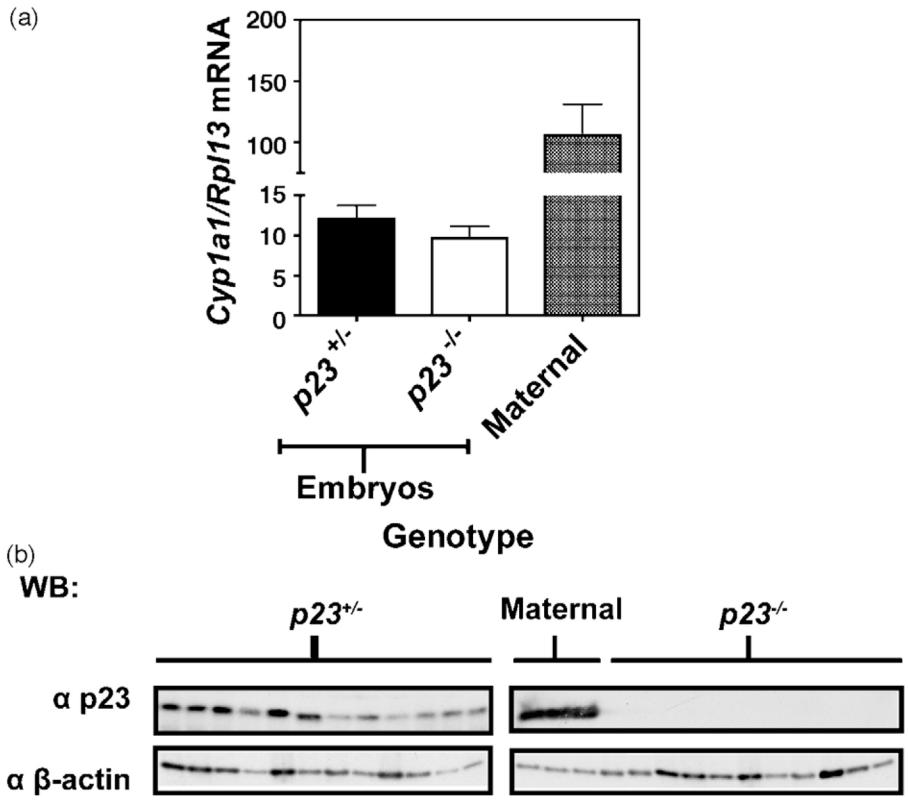

Fig. 4.

p23 expression is not necessary for AHR ligand activation. (a) β-NF induced hepatic *Cyp1a1* mRNA expression in both $p23^{-/-}$ and $p23^{+/-}$ murine embryos and maternal tissue. Females were examined each morning, and those with copulatory plugs were considered to be at gestational day 0.5. Pregnant $p23^{+/-}$ dams were treated with 20 mg/kg β-NF in olive oil for 6 h, embryonic and maternal livers were harvested (gestational day 18) and RNA was isolated. Levels of *Cyp1a1* mRNA expression were measured using real-time RT-PCR. (b) The presence of p23 and β-actin (control) was assessed using western blot analysis.