Short Communication

Leflunomide Induces Pulmonary and Hepatic CYP1A Enzymes via Aryl Hydrocarbon Receptor^S

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ABSTRACT

Emerging evidence indicates that the aryl hydrocarbon receptor (AhR) plays a crucial role in normal physiologic homeostasis. Additionally, aberrant AhR signaling leads to several pathologic states in the lung and liver. Activation of AhR transcriptionally induces phase I (CYP1A) detoxifying enzymes. Although the effects of the classic AhR ligands such as 3-methylcholanthrene and dioxins on phase 1 enzymes are well studied in rodent lung, liver, and other organs, the toxicity profiles limit their use as therapeutic agents in humans. Hence, there is a need to identify and investigate nontoxic AhR ligands not only to understand the AhR biology but also to develop the AhR as a clinically relevant therapeutic target. Leflunomide is a Food and Drug Administration–approved drug in humans that is known to have AhR agonist activity in vitro. Whether

it activates AhR and induces phase 1 enzymes in vivo is unknown. Therefore, we tested the hypothesis that leflunomide will induce pulmonary and hepatic CYP1A enzymes in C57BL/6J wild-type mice, but not in AhR-null mice. We performed real-time reverse-transcription polymerase chain reaction analyses for CYP1A1/2 mRNA expression, western blot assays for CYP1A1/2 protein expression, and ethoxyresorufin-O-deethylase assay for CYP1A1 catalytic activity. Leflunomide increased CYP1A1/A2 mRNA, protein, and enzymatic activities in wild-type mice. In contrast, leflunomide failed to increase pulmonary and hepatic CYP1A enzymes in AhR-null mice. In conclusion, we provide evidence that leflunomide induces pulmonary and hepatic CYP1A enzymes via the AhR.

Introduction

The aryl hydrocarbon receptor (AhR) is a member of the basic helix-loop-helix/PER-ARNT-SIM family of transcriptional regulators (Burbach et al., 1992). AhR activation results in a conformational change of the cytosolic AhR complex that exposes the nuclear localization sequence(s), resulting in translocation of this complex into the nucleus (Hord and Perdew, 1994; Pollenz et al., 1994). In the nucleus, AhR dissociates from the core complex, dimerizes with the AhR nuclear translocator, and initiates transcription of phase I enzymes such as CYP1A (Nebert et al., 2004). The cytochrome P450 enzymes belong to a superfamily of heme proteins that are involved in the metabolism of exogenous and endogenous chemicals (Guengerich, 1990). The mammalian CYP1A subfamily has two isoforms, CYP1A1 and CYP1A2. CYP1A1 is essentially an extrahepatic enzyme, whereas CYP1A2 is expressed mainly in the liver, and is not or is weakly expressed in extrahepatic tissues. AhR is of particular interest to toxicologists, and extensive research has been conducted on its role in the bioactivation of polycyclic and aromatic hydrocarbons leading to carcinogenesis (Nebert et al., 2004). However, the creation of knockout and transgenic mice has provided mechanistic insights into the potential role(s) that AhR might play in normal physiologic homeostasis (Bock and Kohle, 2009; Fujii-Kuriyama and Kawajiri, 2010; Sauzeau et al., 2011; Lindsey and Papoutsakis, 2012). Hence, a search for novel and

nontoxic AhR agonists is of paramount importance to understand the role of AhR and its downstream target genes, such as CYP1A enzymes, in normal physiologic and abnormal disease states. To this end, we chose leflunomide, a Food and Drug Administration–approved drug in humans as well as an AhR agonist, to test our hypothesis.

Leflunomide, or *N*-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide (Supplemental Fig. 1), is an immunomodulatory drug that is used to treat humans with rheumatoid arthritis (Pinto and Dougados, 2006). The immunomodulatory activity of leflunomide is mediated by its primary metabolite, teriflunomide, which inhibits dihydroorotate dehydrogenase, a mitochondrial enzyme involved in de novo pyrimidine synthesis (Teschner and Burst, 2010). Leflunomide is known to activate AhR and its downstream target genes, such as CYP1A enzymes, mainly in vitro (O'Donnell et al., 2010, 2012; Jin et al., 2012, 2014). An elegant study by Hu et al. (2007) demonstrated that leflunomide directly binds to and activates AhR in vitro and induces hepatic CYP1A mRNA in vivo in rats. However, in vivo studies are necessary to determine the role of leflunomide as an AhR agonist in various other organs, such as lungs, so that it can be considered as a therapeutic option for both lung and liver disorders. Thus, the goal of this study was to investigate the effects of leflunomide on AhR-mediated expression of pulmonary and hepatic CYP1A enzymes. Using C57BL/6J wild-type (WT) and AhR-null mice, we tested the hypothesis that leflunomide will induce pulmonary and hepatic CYP1A enzymes in WT mice, but not in AhR-null mice.

Materials and Methods

Animals. The C57BL/6J WT mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Dr. Christopher A. Bradfield (University of Wisconsin, Madison, WI) provided the AhR-null mice on a C57BL/6J

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; EROD, ethoxyresorufin-O-deethylase; L 40, leflunomide 40 mg/kg/day; MC, methylcholanthrene; PEG, polyethylene glycol; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; WT, wild type.

background. Ten-week-old female WT and AhR-null mice maintained at the Texas Children's Hospital animal facility were used for the study.

Chemicals. Leflunomide was purchased from Sigma-Aldrich (St. Louis, MO). Buffer components for electrophoresis were obtained from Bio-Rad (Hercules, CA).

Experiment Design. Ten-week-old WT female mice were injected i.p. with either 20 or 40 mg/kg/day leflunomide (L 40; $n = 3$ /dose) or vehicle, polyethylene glycol (PEG) (controls, $n = 3$) once daily for 3 days. The AhR-null female mice (10-week-old) were injected intraperitoneally with vehicle, PEG ($n = 3$), or L 40 ($n = 3$) once daily for 3 days. We administered leflunomide parenterally (i.p.) to determine its feasibility as an AhR agonist in sick patients who are unable to take medications by mouth. Additionally, WT female mice were injected i.p. with either 26 mg/kg/d 3-methylcholanthrene (MC; $n = 3$) or vehicle, PEG ($n = 3$), once daily for 3 days to compare the potency of leflunomide with a prototypical CYP1A inducer such as MC. After the experiments, the lung and liver tissues were harvested for analysis of CYP1A induction.

Real-Time Reverse-Transcription Polymerase Chain Reaction Assays. Total mRNA was isolated and reverse transcribed to cDNA as mentioned before (Shivanna et al., 2011a). Real-time quantitative reverse-transcription polymerase chain reaction analysis was performed with the 7900HT Real-Time PCR System using iTaq Universal SYBR Green Supermix (1725121; Bio-Rad). The sequences of the primer pairs were *mCYP1a1*: 5'-GGT TAA CCA TGA CCG GGA ACT-3' and 5'-TGC CCA AAC CAA AGA GAG TGA-3'; *mCYP1a2*: 5'-TGG AGC TGG CTT TGA CAC AG-3' and 5'-CGT TAG GCC ATG TCA CAA GTA GC-3'; and *m β -actin*: 5'-TAT TGG CAA CGA GCG GTT CC-3' and 5'-GGC ATA GAG GTC TTT ACG GAT GTC-3'. β -Actin was used as the reference gene. Following a reverse-transcription hold for 10 minutes at 95°C, the samples were denatured at 95°C for 10 minutes. The thermal cycling step was for 40 cycles at 95°C for 15 seconds, and 40 cycles at 60°C for 1 minute (Anwar-Mohamed et al., 2012).

Preparation of Microsomes and Enzyme Assays. Lung and liver microsomes were isolated as described previously (Moorthy et al., 1997; Jiang et al., 2004). Ethoxoresorufin-*O*-deethylase (EROD; CYP1A1) activity in lung microsomes was assayed as mentioned before (Moorthy et al., 1997; Jiang et al., 2004).

Western Blotting. Lung and liver microsomal proteins prepared from individual animals were subjected to SDS polyacrylamide gel electrophoresis.

The separated proteins on the gels were transferred to polyvinylidene difluoride membranes, followed by western blotting. The membranes were incubated overnight at 4°C with the following primary antibodies: anti-CYP1A1 (which cross-reacts with CYP1A2), a specific anti-CYP1A2 (dilution 1:1500; CYP1A1 and A2 antibodies were gifts from P.E. Thomas, Rutgers University, Piscataway, NJ), and anti-GRP 78 (sc-13968, dilution 1:500; Santa Cruz Biotechnologies, Santa Cruz, CA) antibodies. The immuno-reactive bands were detected by chemiluminescence methods.

Analyses of Data. The results were analyzed by GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). At least three separate experiments were performed in WT mice for each measurement (n = total animals from one of the three experiments), and the data are expressed as means $\pm$ S.E.M. The effects of leflunomide and AhR gene on the expression of CYP1A enzymes were assessed using analysis of variance techniques and t tests. Multiple comparison testing by the post-hoc Bonferroni test was performed if statistical significance was noted by analysis of variance. A P value <0.05 was considered significant.

Results and Discussion

This study demonstrates that leflunomide induces the expression of the phase I enzymes, CYP1A1/A2, via the AhR. In the adult WT mice in vivo, leflunomide induced CYP1A1/A2 expression when compared with controls, whereas in adult AhR-null mice, the lack of leflunomide-mediated induction of CYP1A1/A2 enzymes correlated with the absence of a functional AhR gene.

The AhR is a versatile transcription factor that has important physiologic functions in addition to its widely established role in xenobiotic metabolism. Studies from our and other laboratories have reported that AhR and its downstream target, CYP1A enzymes, may be a crucial regulator of pulmonary and hepatic oxidant stress and inflammation in mice through the induction of several detoxifying enzymes or via "cross-talk" with other signal transduction pathways (Jiang et al., 2004; Thatcher et al., 2007; Baglolle et al., 2008; Rico de

Fig. 1. Leflunomide induces pulmonary CYP1A1 enzyme in WT mice. The lungs of WT mice treated with vehicle (PEG), 20 mg/kg of leflunomide (L 20) or L 40, or 26 mg/kg/day MC i.p. daily for 3 days were harvested to determine pulmonary CYP1A1 protein expression (A), enzyme activity (C), and mRNA expression (D). Densitometric analyses wherein CYP1A1 (B) band intensities were normalized to GRP 78. PC, positive control (MC-treated WT mice). Values are means $\pm$ S.E.M. from three individual animals in each group from one experiment. *Significant differences between PEG and leflunomide groups ($P < 0.05$); †significant differences between the leflunomide groups ($P < 0.05$).

Souza et al., 2011; Shivanna et al., 2011b, 2013). However, the prototypical inducers such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and MC are unsuitable for clinical use because of their well known toxicities. Hence, identification of novel nontoxic AhR ligands is important for developing the AhR as a clinically relevant therapeutic target in lung and liver disorders mediated by oxidant injury and inflammation. Hence, we conducted *in vivo* studies with a novel Food and Drug Administration-approved drug, leflunomide, to determine whether it induces the expression of the AhR-regulated pulmonary and hepatic CYP1A enzymes.

The dose of leflunomide used in this study was comparable to the dose used in previous rodent studies (Hu et al., 2007; Baban et al.,

2012). Initially, we determined the effects of leflunomide on pulmonary and hepatic CYP1A apoprotein expression in WT mice. Consistent with our prior studies, only CYP1A1 apoprotein was expressed in the lungs (Fig. 1A), whereas both CYP1A1 and 1A2 apoproteins were expressed in the liver (Fig. 2A). Interestingly, leflunomide increased pulmonary and hepatic CYP1A1 apoprotein expression, wherein 20 or 40 mg/kg/day leflunomide induced pulmonary CYP1A1 apoprotein (Fig. 1, A and B) and 40 mg/kg/day leflunomide was required to induce hepatic CYP1A1 apoprotein (Fig. 2A) expression. Immunoblotting with a specific CYP1A2 antibody showed that leflunomide at either dose induced hepatic CYP1A2 apoprotein (Fig. 2, B and C). Next, we determined the effects of leflunomide on CYP1A enzyme activities.

Fig. 2. Leflunomide induces hepatic CYP1A enzymes in WT mice. The liver tissues of WT mice treated as mentioned in Fig. 1 were harvested to determine hepatic CYP1A1 (A) and A2 (B) protein expression, EROD (D) activity, and CYP1A1 (E) and CYP1A2 (F) mRNA expression. Densitometric analyses wherein CYP1A2 (C) band intensities were normalized to GRP 78. Values are means $\pm$ S.E.M. from three individual animals in each group from one experiment. *Significant differences between PEG and leflunomide groups ($P < 0.05$); †significant differences between the leflunomide groups ($P < 0.05$). L 20, 20 mg/kg of leflunomide; PC, positive control (MC-treated WT mice).

Fig. 3. Leflunomide fails to induce pulmonary and hepatic CYP1A enzymes in AhR-null mice. The tissues of AhR-null mice treated with PEG or L 40 i.p. daily for 3 days were harvested to determine pulmonary and hepatic CYP1A1 protein expression (A and D), enzyme activity (B and E), mRNA expression (C and F), and hepatic CYP1A2 protein (D) and mRNA (G) expression. PC, positive control (MC-treated WT mice). Values are means $\pm$ S.E.M. from three individual animals in each group. PC, positive control (MC-treated WT mice).

Leflunomide at a dose of 40 mg/kg/day increased pulmonary (Fig. 1C) and hepatic (Fig. 2D) EROD activities in WT mice, which indicates induction of pulmonary and hepatic CYP1A1 expression, as EROD activity is relatively specific for CYP1A1 enzyme (Burke et al., 1994; Moorthy et al., 2000; Couroucli et al., 2002). In regard to CYP1A2, 40 mg/kg/day leflunomide induced hepatic CYP1A2 apoprotein (Fig. 2, B and C) and CYP1A2 mRNA (Fig. 2F) expression. Hu et al. (2007) noticed a similar increase of hepatic CYP1A1 and CYP1A2 mRNA expression in adult rats treated with leflunomide. However, the knowledge gaps of the mechanism of hepatic CYP1A1/A2 induction by leflunomide and the effects of leflunomide on hepatic CYP1A1/A2 apoprotein expression and enzyme activities and pulmonary CYP1A expression in vivo are addressed in the current investigation. The elegant in vitro study by O'Donnell et al. (2010) indicates that leflunomide, but not its metabolite, teriflunomide, activates AhR. This suggests that leflunomide modulates AhR activation and the immune system via distinct mechanisms, and the rate of metabolism of leflunomide is a critical determinant of AhR activation. MC at 26 mg/kg is a more effective pulmonary [CYP1A1 enzyme activity: 1344.86 vs. 464.01 (L 40) pmol/min/mg (Fig. 1C), and CYP1A1 mRNA expression: 2331.76- vs. 29.93-fold (L 40) increase (Fig. 1D)] and hepatic [CYP1A1 enzyme activity: 8977.9 vs. 3524.74 (L 40) pmol/min/mg (Fig. 2D), CYP1A1 mRNA expression: 10,189.6- vs. 64.48-fold (L 40) increase (Fig. 2E), and CYP1A2 mRNA expression: 35.35- vs. 5.34-fold (L 40) increase (Fig. 2F)] CYP1A inducer than leflunomide at 40 mg/kg.

The mechanistic role of AhR in the induction of CYP1A by prototypical inducers, MC and TCDD, has been extensively studied. Whether leflunomide induces CYP1A enzymes via AhR in vivo is unknown. Therefore, we conducted experiments with leflunomide in AhR-null mice to delineate the precise role of AhR in leflunomide-mediated induction of CYP1A enzymes. In AhR-null mice, leflunomide failed to enhance pulmonary (Fig. 3, A–C) and hepatic (Fig. 3, D–G) CYP1A1/A2 mRNA and apoprotein expression and EROD activity, supporting the hypothesis that induction of pulmonary and hepatic CYP1A enzymes by leflunomide is mediated by AhR-dependent mechanisms. To the best of our knowledge, this is the first in vivo study to demonstrate that AhR is critical for the upregulation of pulmonary and hepatic CYP1A1/A2 enzymes by leflunomide in mice.

In summary, we provide evidence that leflunomide induces pulmonary and hepatic CYP1A1 and hepatic CYP1A2 enzymes in vivo via an AhR-mediated mechanism. Our results suggest that leflunomide can be used to investigate AhR biology in the lung and liver, which can lead to the discovery of novel therapies in the prevention and treatment of oxidative stress- and inflammation-induced disorders, such as bronchopulmonary dysplasia in premature infants and acute respiratory distress syndrome, chronic obstructive pulmonary disease, and malignancies in adults.

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