Role of constitutive androstane receptor in Toll-like receptor-mediated regulation of gene expression of hepatic drug metabolizing enzymes and transporters.

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Abbreviations: CAR, constitutive androstane receptor; PXR, pregnane X receptor; RXR, retinoid X receptor; NRs, nuclear receptors; LTA, lipoteichoic acid; LPS, lipopolysaccharide; PB, Phenobarbital, TLRs, Toll-like receptors; DMEs, drug metabolizing enzymes; cytochrome P450, CYP; TCPOBOP, 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene.
Abstract:

Impairment of drug disposition in the liver during inflammation has been attributed to down-regulation of gene expression of drug metabolizing enzymes (DMEs) and drug transporters. Inflammatory responses in the liver are primarily mediated by toll-like receptors (TLRs). We have recently shown that activation of TLR2 or TLR4 by lipoteichoic acid (LTA) and lipopolysaccharide (LPS), respectively, leads to the down-regulation of gene expression of DMEs/transporters. However, the molecular mechanism underlying this down-regulation is not fully understood. The xenobiotic nuclear receptors, pregnane-X-receptor (PXR) and constitutive androstane receptor (CAR) regulate the expression of DMEs/transporter genes. Down-regulation of DMEs/transporters by LTA or LPS was associated with reduced expression of PXR and CAR genes. To determine the role of CAR, we injected, CAR+/+ and CAR−/− mice with LTA or LPS, which significantly down-regulated (~40-60%) RNA levels of the DMEs, Cyp3a11, Cyp2a4, Cyp2b10, Ugt1a1, Sultn and the transporter, Mrp2 in CAR+/+ mice. Suppression of most of these genes was attenuated in LTA-treated CAR−/− mice. In contrast, LPS-mediated down-regulation of these genes was not attenuated in CAR−/− mice. Induction of these genes by mouse CAR activator 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) was sustained in LTA but not in LPS-treated mice. Similar observations were obtained in humanized CAR mice. We have replicated these results in primary hepatocytes as well. Thus LPS can down-regulate DME/transporter genes in the absence of CAR whereas, the effect of LTA on these genes is attenuated in the absence of CAR, indicating the potential involvement of CAR in LTA-mediated down-regulation of DME/transporter genes.
Introduction:

Impairment of drug disposition during inflammation is attributed to down-regulation of gene expression of phase I and phase II drug metabolizing enzymes (DMEs) and transporters (Sewer et al., 1997; Renton & Nicholson, 2000). The modulation of DMEs and transporters at the transcriptional level is regulated by basal transcription factors as well as the xenobiotic nuclear receptors (NRs), pregnane-X-receptor (PXR) and constitutive androstane receptor (CAR). The NRs heterodimerize with retinoid-X-receptor (RXR)α to bind to the promoter regions of the target genes (Shen & Kong, 2009; Zordoky & El-Kadi, 2009; Mangelsdorf et al., 1995; Kakizaki et al., 2008; Tien & Negishi, 2006). The molecular mechanism by which DMEs and transporters are down-regulated during inflammation is not fully understood.

Toll-like receptors (TLRs) are the major mediators of inflammatory responses in the liver, and recognize microbial components as well as endogenous ligands from damaged or stressed cells (Takeda & Akira, 2001; Ishii & Akira, 2004; Schwabe et al., 2006). TLRs are present on non-parenchymal cells including Kupffer cells as well as on hepatocytes in the liver (Liu et al., 2002; Matsumura et al., 2003). We have shown that activation of TLR4 by LPS (gram-negative bacterial component) down-regulates the gene expression of select DMEs and transporters (Ghose et al., 2008). LPS-treated rodents are well-established models of inflammation, and LPS treatment induces pro-inflammatory cytokines, interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)α. These cytokines act on hepatocytes to reduce the expression of DME/transporter genes. (Renton KW., 2004; Aitken et al., 2006). We have also shown that activation of TLR2 by LTA (gram-positive bacterial component) down-regulates the expression of select DME and transporter genes (Ghose et al., 2009, 2011).
Several studies have shown that down-regulation of PXR and CAR genes was associated with decreased DME and transporter gene expression (Beigneux et al., 2000, 2002; Ghose et al., 2004, 2008, 2009). We have also shown that there is preferential suppression of CAR and its target hepatic genes on administration of LTA (Ghose et al., 2009). Thus, it is likely that nuclear receptors play an important role in inflammation-mediated down-regulation of gene expression of DMEs and transporters.

Studies in PXR$^{-/-}$ mice showed that PXR is not responsible for the regulation of cytochrome P450 (Cyp) genes during LPS-mediated inflammation (Richardson & Morgan, 2005; Hartmann et al., 2001). On the other hand, PXR was shown to be involved in down-regulation of Cyp3a11, multi drug resistance-associated protein (Mrp)2 and bile salt exporter pump in IL-6 treated mice (Teng & Piquette-Miller, 2005).

The goal of this paper is to determine the role of CAR in down-regulation of DMEs and transporters by comparing the effects of LTA or LPS in CAR$^{+/+}$, CAR$^{-/-}$ and CAR-activated mice (treated with mouse CAR activator, TCPOBOP). We find that LTA administration led to significant down-regulation of gene expression of key DMEs, Cyp3a11, Cyp2a4, Cyp2b10, uridine diphosphate glucuronosyltransferase (Ugt)1a1, amine N-sulfotransferase (Sultn), and the transporter, Mrp2 in CAR$^{+/+}$ mice. Down-regulation of Cyp3a11, Cyp2a4, Cyp2b10 and Ugt1a1 was completely attenuated whereas the down-regulation of Sultn and Mrp2 was attenuated at certain time-points in LTA-treated CAR$^{-/-}$ mice. On the other hand, LPS administration led to significant down-regulation of Cyp3a11, Cyp2a4, Ugt1a1 and Mrp2 in CAR$^{+/+}$ as well as CAR$^{-/-}$ mice. Activation of mouse CAR with TCPOBOP increased nuclear CAR protein levels and induced the expression of all the above-mentioned genes as expected. Surprisingly, the induction of all these genes, and increased CAR protein levels in the nucleus was still detected in the
presence of LTA. On the other hand, the induction of these genes by TCPOBOP was attenuated in the presence of LPS, along with attenuation of CAR protein levels in the nucleus. We have replicated the above results in primary hepatocytes as well. These results were also confirmed in humanized CAR (hCAR) mice treated with the universal CAR activator, PB.

The outcome of these studies demonstrates that CAR is required for down-regulation of hepatic DME/transporter genes by LTA, but LPS can down-regulate these genes in the absence of CAR. Surprisingly, induction of CAR-mediated genes in TCPOBOP-treated mice was maintained in LTA, but not in LPS-injected mice. This was likely due to the inability of LTA, in the presence of TCPOBOP to down-regulate gene, and consequently protein expression of CAR. These results indicate that differential CAR expression may contribute to the regulation of hepatic DMEs and transporters upon infections by gram-positive and gram-negative bacterial components.
Materials & Methods:

Materials.

Highly purified lipoteichoic acid (*Staphylococcus aureus*) and lipopolysaccharide (*Escherichia coli*) were purchased from InvivoGen (San Diego, U.S.A) and freshly diluted to the required concentration in 0.9% saline. The sequences of the primers and probes were obtained from the literature as reported previously (Ghose et al., 2004, 2009, 2011). All oligonucleotides were purchased from Sigma Genosys (Houston, U.S.A) and all real-time PCR reagents were purchased from Applied Biosystems, (Foster city, CA, U.S.A.). Rabbit anti-CAR antibody (#sc-8538), lactate dehydrogenase (LDH) (#sc-33781) and lamin A/C (#sc-20681) (Santa Cruz biotechnology CA, U.S.A) were used as per the manufacturer’s instructions. Cell culture media and media supplements were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). Unless specified, all other materials were purchased from Sigma-Aldrich (St Louis, MO, USA.).

Animals and treatments.

Adult, male, (~8 weeks old, 20-25g) CAR⁻/⁻ mice (Wei et al., 2000) and the corresponding wild-type mice on a C57BL/6 background were maintained in a 12 h dark/light cycle and in a temperature-and-humidity-controlled environment. The mice had access to regular rodent chow and water *ad libitum*. The mice were intraperitoneally (i.p.)-injected with LTA (6 mg/kg b.w) or LPS (2 mg/kg b.w) and the vehicle, saline. Livers were harvested at various time-points for RNA and protein analysis. In order to activate CAR, mice were i.p.-injected with 3 mg/kg b.w of TCPOBOP i.p. in corn oil for 3 days (Baskin-Bey et al., 2006) or PB (80mg/kg/day, i.p. in saline) for 3 days prior to LTA or LPS treatment. The hCAR mice used were generated using the knock-in strategy as described previously (Zhang et al., 2002). All the animal care and use
protocols were approved by the Institutional Animal Care and Use Committee guidelines. All experiments were performed in triplicate and repeated three to four times.

**Real-Time PCR.**

Total RNA was isolated from mouse liver using TRIzol reagent (Sigma-Aldrich, St Louis, MO, U.S.A) according to the manufacturer’s protocol. cDNA was synthesized using the High Capacity Reverse Transcription Kit from Applied Biosystems. Real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System instrument and software (Applied Biosystems) as described previously (Ghose et al., 2004). In short, each reaction mixture (total of 25μl) contained 50-100 ng of cDNA, 300 nM forward primer, 300 nM reverse primer, 200 nM fluorogenic probe, and 15 μl of TaqMan Universal PCR Master Mix. We extrapolated the quantitative expression values from standard curves and these values were normalized to cyclophilin.

**Primary hepatocyte isolation and treatment.**

Primary mouse hepatocytes were isolated from CAR^+/+ and CAR^-/- mice using the two step collagenase perfusion technique as described previously (Li et al., 2002; Ghose et al., 2011). In short, after digestion, the liver was excised and then the hepatocytes were purified using a percoll gradient (33%), washed and screened for viability using trypan blue exclusion technique. Only isolations with viability of more than 90% were used for these studies. Cells were plated at a density of 500,000 cells per well in six-well Primaria plates (BD biosciences, San Diego, CA, U.S.A) and allowed to attach for 4 h. Cells were maintained for 48 h with daily change of medium. The cells were incubated with serum-free Williams medium E two hours prior to treatment with 50 ng/ml LTA (8 h) or 1 μg/ml LPS (16 h). In order to activate CAR, the cells
were treated with 250 nM of TCPOBOP 24 h or DMSO (0.025%v/v) prior to treatment with LTA or LPS. RNA was then isolated for real-time PCR analysis as described above.

**Immunoblotting.**

Nuclear extracts were prepared as described previously (Ghose et al., 2004) and the protein concentration was determined using the bicinchoninic acid (BCA) assay according to the manufacturer’s protocol (Pierce, Rockford, IL, U.S.A). Equal amounts of protein (10 μg) were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were then probed with rabbit anti-CAR (1:500), anti Lamin A/C (1:500) or anti-LDH (1:500) antibody followed by probing with a goat anti-rabbit IgG-AP secondary antibody (1:2000). The membranes were then washed and incubated with Tropix® CDP star® nitroblock II™ ECL reagent as per the manufacturers’ instructions (Applied Biosystems, CA, U.S.A.). The membranes were analyzed using FlourChem FC imaging system (Alpha Innotech). The images were quantified by densitometer using AlphaEase software.

**Statistical Analysis.**

Treatment groups were compared using 2 way ANOVA followed by a post-hoc test (tukey’s post hoc test) with p < 0.05. The results were presented as mean ± SD.
RESULTS:

Effect of LTA treatment on DME/transporter gene expression in CAR^{+/+} and CAR^{−/−} mice.

We have shown previously that LTA causes preferential suppression of CAR and its target genes (Ghose et al., 2009). To investigate the role of CAR in LTA-mediated down-regulation of DMEs and transporters, CAR^{+/+} and CAR^{−/−} mice were injected with LTA. RNA was isolated from the livers harvested at the indicated time points and analyzed by real-time PCR. RNA levels of the key phase I enzyme, Cyp3a11 were down-regulated at 4-16 h (~30-40%) in the CAR^{+/+} mice after LTA administration, whereas this down-regulation was absent in CAR^{−/−} mice. The basal expression of Cyp3a11 is significantly lower (~50-60%) in the CAR^{−/−} mice. This observation is in agreement with other reports published previously (Assem et al., 2004). Cyp2a4 RNA levels were significantly down-regulated in CAR^{+/+} mice at 4 and 8 h (~60-80%), whereas this down-regulation was attenuated in the CAR^{−/−} mice. As seen previously (Ghose et al., 2009), the RNA levels of Cyp2b10 in CAR^{+/+} mice were down-regulated at 4 h (~75-80%) and remained reduced (~40%) until 16 h after LTA administration (Fig. 1). The basal expression of Cyp2b10 in the CAR^{−/−} mice is too low to make any conclusive inference.

In the case of phase II enzymes, Ugt1a1 RNA levels were significantly (~40%) reduced at 16 h in CAR^{+/+} mice treated with LTA, while this reduction was attenuated in the CAR^{−/−} mice. Ugt1a1 RNA levels remained unchanged at 4 h and 8 h in LTA-treated CAR^{+/+} and CAR^{−/−} mice. Sultn RNA levels were down-regulated in CAR^{+/+} mice at 4 and 8 h (~40-55%), while no reduction was seen at 16 h. The down-regulation of Sultn in CAR^{−/−} mice was not detected at 4 h and 8 h of LTA treatment, though there was down-regulation of Sultn at 16 h (~60%). This suggests delayed action of LTA on Sultn in the absence of CAR, and/or the existence of alternate mechanisms.
RNA levels of the transporter, Mrp2 were down-regulated (~40%) by LTA at 4 h in both CAR\(^{+/+}\) and CAR\(^{-/-}\) mice, whereas at 8 h, Mrp2 down-regulation (~35%) was detected only in the CAR\(^{+/+}\) mice. Mrp2 gene expression was unaffected at 16 h in both CAR\(^{+/+}\) and CAR\(^{-/-}\) mice treated with LTA. We also examined RNA levels of two other transporter genes namely Mrp4 (unchanged in CAR\(^{+/+}\) and CAR\(^{-/-}\) mice) and Mdr1b (up-regulated in both CAR\(^{+/+}\) and CAR\(^{-/-}\) mice) (data not shown). This finding indicates that basal expression of CAR is required for LTA to reduce DME and transporter genes.

Effect of LPS treatment on DME/transporter gene expression in CAR\(^{+/+}\) and CAR\(^{-/-}\) mice

LPS administration significantly down-regulated RNA levels of the phase I enzymes (Cyp3a11, Cyp2a4), the phase II enzyme, Ugt1a1 and the transporter, Mrp2 in both CAR\(^{+/+}\) and CAR\(^{-/-}\) mice at 16 h (Fig. 2). Amongst all the genes studied, Cyp3a11 was the most affected (~80% reduction) followed by Cyp2a4 (~65% reduction), Ugt1a1 (~50% reduction) and Mrp2 (~40% reduction). Since, LPS down-regulates DME/transporter genes in the absence of CAR, these results indicate that CAR is not required for LPS-mediated down-regulation of DME and transporter genes. Cyp2b10 and Sultn mRNA levels remained unchanged in both CAR\(^{+/+}\) and CAR\(^{-/-}\) mice. We selected the 16 h time point because we saw optimal down-regulation of these DMEs and transporters at this time-point (Ghose et al., 2004).

Effect of LTA or LPS treatment on DME/transporter gene expression in TCPOBOP-treated Mice.

To further confirm the role of CAR in LTA-mediated down-regulation of DME/transporter genes, we activated CAR using specific mouse CAR activator TCPOBOP (3 mg/kg/day in corn oil i.p.) 3 days prior to administration of LTA (Fig. 3). TCPOBOP treatment caused a significant
induction of Cyp3a11, Cyp2a4, Cyp2b10, Ugt1a1, Sultn and Mrp2 genes (Fig. 3A; 3B). Since we saw maximum down-regulation of DMEs and transporters by LTA at 4 h, we treated these TCPOBOP treated CAR\(^{+/+}\) mice with LTA for 4 h. Surprisingly, this induction was still detected in the presence of LTA. LTA treatment at longer time-points (8-16 h) did not affect the induction of these DMEs/transporters by TCPOBOP (data not shown). Since LTA down-regulated Ugt1a1 at 16 h in CAR\(^{+/+}\) mice (Fig. 1), we analyzed mRNA levels of Ugt1a1 after 16 h treatment with LTA in the TCPOBOP-treated mice (Fig. 3B). As seen with other DMEs, induction of Ugt1a1 RNA levels by TCPOBOP was not affected by LTA treatment. Similar results were observed by treatment of mice with the universal CAR activator, PB (data not shown).

In order to understand the mechanism underlying the lack of effect of LTA on TCPOBOP-mediated induction of DME/transporter genes, we measured expression of CAR at the gene and protein levels in the presence of TCPOBOP. As observed in our previous publications (Ghose et al., 2009), we found that CAR gene expression and cytosolic protein levels were reduced by LTA in the absence of TCPOBOP (data not shown); nuclear levels of CAR were too low to obtain a conclusive inference.

TCPOBOP, by itself caused increased accumulation of CAR in the nucleus with no effect on CAR gene expression as expected. Interestingly, we did not observe any changes in CAR gene expression and nuclear/cytosolic levels by LTA in the presence of TCPOBOP (Fig. 3 C, D & E). Presence of high amount of CAR in TCPOBOP/LTA-treated mice may account for increased expression of CAR-target genes in these mice compared to corn oil/LTA-treated controls.
We find that LPS down-regulated the DME and transporter genes to the same extent in mice pre-treated with TCPOBOP or the vehicle control, corn oil (Fig. 4A). We also found that CAR gene expression as well as nuclear/cytosolic protein levels were down-regulated by LPS to the same extent in CAR \(^{+/+}\) and TCPOBOP-treated mice (Fig. 4 B, C & D). As shown in our previous publications (Ghose et al., 2004), LPS down-regulated CAR expression in the absence of TCPOBOP (data not shown).

**Effect of LTA or LPS treatment on DME/transporter gene expression in PB-treated hCAR mice.**

In order to test the relevance of these findings on human CAR, we injected hCAR mice with PB (80 mg/kg i.p.) for 3 days, prior to administration of LTA or LPS. LTA treatment led to down-regulation of gene expression of Cyp3a11 (~45%), Cyp2a4 (~65%), Cyp2b10 (~60%), Ugt1a1 (~50%), Sultn (~55%) and Mrp2 (~50%) (Fig. 5A and B). PB treatment significantly induced the expression of these genes, while LTA treatment did not attenuate this induction (Fig. 5A and B). On the other hand, LPS administration in hCAR mice led to down-regulation of gene expression of Cyp3a11 (~80%), Cyp2a4 (~60%), Ugt1a1 (~55%) and Mrp2 (~45%) (Fig. 5C). Interestingly, LPS down-regulated the gene expression of these genes to the same extent in mice pre-treated with or without PB (Fig. 5C). This data is in agreement with the previous findings in LTA or LPS-injected CAR \(^{+/+}\) mice pre-treated with TCPOBOP.

**Effect of LTA or LPS treatment on DME/transporter gene expression in primary hepatocytes from CAR \(^{+/+}\) and CAR \(^{−/−}\) mice.**

The role of CAR was further examined in primary hepatocytes treated with saline or LTA (50 ng/ml) for 8 h. RNA levels of Cyp3a11, Cyp2a4, Cyp2b10 and Ugt1a1 were significantly down-
regulated (~40-45%) in LTA-treated CAR<sup>+/+</sup> hepatocytes, and this down-regulation was attenuated in CAR<sup>−/−</sup> hepatocytes (Fig. 6A). Sultn gene expression was significantly down-regulated (~40%) in the CAR<sup>+/+</sup> as well as in the CAR<sup>−/−</sup> hepatocytes, however, the extent of down-regulation was lower in the CAR<sup>−/−</sup> hepatocytes (~20%). We did not see any change in the Mrp2 expression either in the CAR<sup>+/+</sup> or CAR<sup>−/−</sup> hepatocytes. As seen in vivo, there were significant differences in the basal levels of Cyp2b10, Cyp3a11 and Ugt1a1 expression between hepatocytes isolated from CAR<sup>+/+</sup> and CAR<sup>−/−</sup> mice. The difference in the trends of Sultn gene expression and Mrp2 gene expression on treatment with LTA is most likely because of the difference in in vitro and in vivo conditions.

CAR<sup>+/+</sup> and CAR<sup>−/−</sup> hepatocytes were treated with saline or LPS (1 μg/ml) for 16 h. Cyp3a11 (~70%), Cyp2a4 (~50%), Ugt1a1 (~60%) and Mrp2 (~50%) were significantly down-regulated in LPS-treated CAR<sup>+/+</sup> as well as in CAR<sup>−/−</sup> hepatocytes (Fig. 6B).

**Effect of LTA or LPS treatment on DME/Transporter gene expression in primary hepatocytes pre-treated with TCPOBOP.**

To activate CAR in vitro, CAR<sup>+/+</sup> hepatocytes were treated with 250 nM TCPOBOP 24 h prior to treatment with saline or LTA (50 ng/ml). TCPOBOP induced the expression of all the DMEs and transporters significantly. As seen in vivo, the down-regulation of DMEs and transporters by LTA was attenuated in hepatocytes treated with TCPOBOP (Fig. 7A).

CAR<sup>+/+</sup> hepatocytes were pre-treated with 250nM TCPOBOP 24 h prior to treatment with LPS (1 μg/ml). LPS administration led to down-regulation of Cyp3a11 (~75%), Cyp2a4 (~50%), Ugt1a1 (~50%) and Mrp2 (~50%). As expected, LPS caused no change in the gene expression of Cyp2b10 or Sultn in hepatocytes treated with TCPOBOP (Fig. 7B). Our in vitro findings concur
with our *in vivo* results suggesting that down-regulation of DME/transporter genes by LPS is associated with down-regulation of gene expression of CAR however; the DME/transporter genes are down-regulated by LPS even in the absence of CAR suggesting the presence of alternative mechanisms. We also performed these experiments in PB-treated primary mouse hepatocytes from hCAR mice and found same results (data not shown).
Discussion:

Infection and inflammation can alter the expression and activities of several of phase I and phase II DMEs and transporters (Cheng & Morgan, 2001; Hartmann et al., 2001). Down-regulation of hepatic DME/transporter genes during inflammation and infection is associated with reduced gene expression of NRs, PXR and CAR. Although, studies have shown that PXR has no role in LPS-mediated regulation of hepatic genes, the role of CAR is not known. In this study, we found that basal levels of CAR are required for LTA to down-regulate the expression of DME/transporter genes. On the other hand, LPS can down-regulate these genes even in the absence of CAR in CAR⁻/⁻ mice.

Our results indicate that both LTA and LPS down-regulate CAR expression, however, they differentially regulate DME and transporter genes. In addition, the time-line of down-regulation of CAR gene expression is different for LTA and LPS, which can partially account for the differential effects on CAR-target genes. LTA down-regulates CAR gene expression (~65%) at 2-4 h, whereas LPS down-regulates CAR gene expression (~70%) at 16 h (Ghose et al., 2008; 2009). LTA administration led to down-regulation of Cyp3a11, Cyp2a4, Cyp2b10, Ugt1a1, Sultn and Mrp2 genes in CAR⁺/⁺ mice at time points ranging from 4-16 h as we have seen previously (Ghose et al., 2009). The reason for discord in the time course of suppression could be the mechanism of down-regulation is different for different genes. Furthermore, LTA may affect corepressor expression and recruitment differently which can lead to differences in the time course of down-regulation of DMEs and transporters. LTA-mediated down-regulation of most of these DME and transporter genes was attenuated in the CAR⁻/⁻ mice suggesting that CAR plays an important role in LTA-mediated down-regulation of these genes.
LPS administration led to down-regulation of Cyp3a11, Cyp2a4, Ugt1a1 and Mrp2 genes in both CAR\(^{+/+}\) and CAR\(^{-/-}\) mice, suggesting that LPS can down-regulate DME/transporter genes in the absence of CAR. It is possible that this LPS-mediated down-regulation may occur through PXR in the absence of CAR. However, we did not find any difference in the expression of PXR between CAR\(^{+/+}\) and CAR\(^{-/-}\) mice treated with saline or LPS (data not shown). This indicates that down-regulation of DME/transporter genes by LPS in CAR\(^{-/-}\) mice are not due to over-expression of PXR. Experiments in PXR/CAR double knockout mice will further explain the roles of PXR and CAR in LTA- or LPS-mediated DME and transporter down-regulation.

Treatment of CAR\(^{+/+}\) mice with specific mouse CAR activator TCPOBOP, results in induction of gene expression of all the DME/transporters mentioned above. Immunoblotting of nuclear extracts from TCPOBOP pre-treated mice revealed that induction of CAR protein levels in the nucleus remained unchanged by LTA treatment (Fig. 3C and D). Furthermore, CAR gene expression in TCPOBOP/LTA-treated mice was significantly higher than corn oil/LTA-injected mice (Fig. 3E).

Since LTA administration in TCPOBOP treated mice did not change gene expression of DMEs and transporters, we administered 12 mg/kg dose of LTA in TCPOBOP-pre-treated mice (double the original dose). However, gene expression of DMEs and transporters still remained unchanged (data not shown). We did not find any down-regulation of DME/transporter genes at different time points (2, 4, 8, 16, 24 and 48 h) in TCPOBOP/LTA-treated mice (data not shown). In order to rule out a ligand dependent effect, we tried another CAR activator PB (80 mg/kg/day i.p. for 3 days) and observed the same results (data not shown).
The mechanism of how LTA down-regulates CAR and its target genes is unclear. We find that activation of CAR with TCPOBOP leads to accumulation of CAR in the nucleus, and LTA treatment does not affect the concentration of nuclear CAR protein levels. Cytosolic CAR protein and CAR gene expression were also unaffected by LTA in the presence of TCPOBOP. Since LTA treatment of CAR−/− mice did not cause down-regulation of DME/transporter genes, we anticipated that induction of CAR by TCPOBOP would facilitate the down-regulation of these genes by LTA. However, in TCPOBOP-treated mice, LTA did not affect DME and transporter gene expression likely due to its lack of effect on CAR gene and protein expression.

It is possible that the induction of DME and transporter genes on administration of TCPOBOP led to enhanced clearance of LTA. Previous studies have shown that LTA is cleared mainly by the liver and kidneys (Hyzy et al., 1992). Thus, the induction of DME/transporter genes may lead to faster clearance of LTA in TCPOBOP pre-treated CAR+/+ mice, leading to diminished effects of LTA on DME/transporter genes.

Activation of TLR2 by its ligands leads to up-regulation of its expression. LTA induced TLR2 expression by ~50 fold in the liver (Ghose et al., 2009), and ~5 fold in odontoblasts (Durand et al., 2006). Another TLR2 ligand, i.e. porin of *Shigella dysenteriae* also induced the expression of TLR2 in hemopoietic cells (Ray et al., 2008). It is possible that induction of TLR2 expression is required for mediating the effects of TLR2 ligands. We find that TLR2 RNA levels were significantly induced by LTA treatment of CAR+/+ and CAR−/− mice (~40-45 fold; Supplemental Fig. 1). Interestingly, TCPOBOP pre-treatment attenuated the induction of TLR2 by LTA. We did not find any induction of TLR2 RNA levels in TCPOBOP/saline-treated mice (Supplemental Fig. 1). Lower TLR2 expression can cause reduced binding of LTA, which can contribute
towards lower in vivo concentration of LTA, thus leading to lack of its effect on DME/transporter genes.

LPS administration in CAR−/− mice led to down-regulation of DME and transporter genes (Fig. 2), hence, we expected that LPS-mediated down-regulation of the genes would be independent of CAR. As expected, LPS treatment led to down-regulation of DMEs and transporters even in mice pre-treated with TCPOBOP (Fig. 4A). Interestingly, TCPOBOP-induced CAR accumulation in the nucleus was significantly attenuated by LPS. Cytosolic CAR protein was also reduced by LPS in TCPOBOP-treated mice. This is likely due to the reduction in CAR gene expression by LPS in the presence of TCPOBOP (Fig. 4 B, C & D). The effect of LPS on CAR nuclear protein levels is contrary to the effects of LTA in TCPOBOP-pre-treated mice, and this is reflected in the different effects of LPS and LTA on DME/transporter genes.

Pro-inflammatory cytokines have been implicated in the down-regulation of hepatic genes during inflammation (Barker et al., 1992; Muntane-Relat et al., 1995). Our results show that the induction of cytokines in the liver by LPS and LTA occurred to different extents. LTA administration led to ~80, 35 and 65 fold induction in IL-6, IL-1β and TNFα respectively, whereas LPS administration led to ~15, 210 and 160 fold inductions (Ghose et al., 2008; 2009). The differential regulation of DME/transporter genes by LTA and LPS may be caused by the differences in induction levels of the cytokines.

LTA treatment induces the pro-inflammatory cytokines, IL-1β, IL-6 and TNFα at 2 h, which coincides with the down-regulation of CAR and its target genes (Ghose et al., 2009). In CAR−/− mice, these cytokines are induced at comparable levels by LTA at 2 h (Supplemental Fig. 2), but DME/transporter genes are not down-regulated by LTA, probably because of the absence of
CAR. IL-6 and IL-1β RNA levels are induced similarly in TCPOBOP pre-treated mice on treatment with LTA, however, the extent of TNFα induction is significantly lower in comparison to CAR<sup>+/+</sup> mice (Supplemental Fig. 2). It is possible that lower expression of TNFα in TCPOBOP/LTA-treated mice accounts for lack of effect of LTA on DME/transporter genes in these mice.

Our study is the first of its kind to determine the role of CAR in regulating DMEs and transporters during inflammation. The down-regulation of DMEs and transporters during inflammation is a complex process, and the molecular mechanism is not fully understood. The effect on hepatic DME/transporter genes depends on the type of inflammatory stimuli, and our results show that there are distinct mechanistic differences between TLR2 and TLR4-induced bacterial inflammation. This indicates that patients exposed to gram-positive and gram-negative infections may have differences in drug disposition due to mechanistic differences in the regulation of DME/transporter genes. Targeting CAR may have therapeutic implications in countering the deleterious effects of gram-positive bacterial infections on hepatic detoxification processes.
Authorship Contributions.

Participated in research design: Ghose and Shah
Conducted experiments: Shah and Guo
Contributed new reagents and analytic tools: Moore
Performed data analysis: Shah and Ghose
Wrote or contributed to the writing of the manuscript: Shah and Ghose
DMD #53850

References:


Footnotes:

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Figure legends:

Fig 1: Regulation of DME and transporter mRNA levels in CAR^{+/-} and CAR^{-/-} mice following LTA administration. CAR^{+/-} and CAR^{-/-} were i.p. injected with saline or LTA (6 mg/kg) and livers were harvested at 4, 8 and 16 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of phase I enzymes, phase II enzymes and transporters were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LTA treatment was compared to the saline-treated controls. * and # indicate significant difference (p < 0.05) between saline and LTA groups in CAR^{+/-} mice and CAR^{-/-} mice respectively and ‡ indicates basal level differences between CAR^{+/-} and CAR^{-/-} mice. The experiments were repeated at least thrice.

Fig 2: Regulation of DME and transporter mRNA levels in CAR^{+/-} and CAR^{-/-} mice following LPS administration. CAR^{+/-} and CAR^{-/-} mice were i.p. injected with saline or 2 mg/kg LPS and livers were harvested at 16 h (n = 5-6 per group). RNA was isolated from the livers and mRNA levels of phase I enzymes, phase II enzymes and transporters were determined by real-time PCR analysis as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LPS treatment was compared to the saline-treated controls. * and # indicate significant difference (p < 0.05) between saline and LPS groups in CAR^{+/-} mice and CAR^{-/-} mice respectively and ‡ indicates basal level differences between CAR^{+/-} and CAR^{-/-} mice. The experiments were repeated at least thrice.
Fig 3: Regulation of DME and transporter mRNA levels in TCPOBOP pre-treated mice following LTA administration. CAR^{+/+} mice were treated with the specific CAR activator, TCPOBOP (3 mg/kg/day) for 3 days prior to i.p. administration of saline or LTA (6 mg/kg). Livers were harvested after: (A) 4 h and (B) 16 h and mRNA levels were analyzed by real-time PCR as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LTA treatment was compared to the saline-treated controls. * indicate significant difference (p < 0.05) between saline and LTA groups and # indicates significant differences between saline samples of -TCPOBOP (-TC) and +TC groups. The experiments were repeated at least thrice. (C) Cytosolic and nuclear extracts were prepared from livers from CAR^{+/+} mice treated with TCPOBOP (3 mg/kg/day) for 3 days prior to saline and LTA (6 mg/kg) i.p. injections (4 h) and CAR protein levels were measured by western blotting. (D) The images were quantified by densitometer using AlphaEase software. The normalized values of fold difference, relative to the expression of LDH for cytosolic extracts and Lamin A/C for nuclear extracts, which was set to 1, are presented as mean ±SD values. (E) Regulation of CAR mRNA levels by LTA in TCPOBOP pre-treated mice. C57BL/6 mice were pre-treated for 3 days with 3 mg/kg TCPOBOP (i.p.) in corn oil prior to treatment with saline or LTA (6 mg/kg) and livers were harvested at 2 h (n = 5 per group). All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in TCPOBOP/saline-treated mice was set to 1, fold change after LTA treatment was compared to the saline-treated controls.

Fig 4: Regulation of DME and transporter mRNA levels in TCPOBOP pre-treated mice following LPS administration. (A) CAR^{+/+} mice were treated with the specific CAR activator
TCPOBOP 3 mg/kg/day for 3 days prior to LPS (2 mg/kg) i.p. administration. Livers were harvested after 16 h and mRNA levels were analyzed by real-time PCR as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LPS treatment was compared to the saline-treated controls. * indicate significant difference ($p < 0.05$) between saline and LPS groups and # indicates significant difference ($p < 0.05$) between saline samples of –TCPOBOP (TC) and +TC groups. The experiments were repeated at least thrice. (B) Cytosolic and nuclear extracts were prepared from livers of CAR$^{+/+}$ mice treated with TCPOBOP 3 mg/kg/day for 3 days prior to saline and LPS (2 mg/kg) i.p. injections (16 h) and CAR protein levels were measured by Western blotting. (C) The images were quantified by densitometer using AlphaEase software. The normalized values of fold difference, relative to the expression of LDH for cytosolic extracts and Lamin A/C for nuclear extracts, which was set to 1, are presented as mean ±SD values. # indicates significant difference ($p < 0.05$) between saline and LPS treatment groups. (D) Regulation of CAR mRNA levels by LPS in TCPOBOP pre-treated mice. C57BL/6 mice were pre-treated for 3 days with 3 mg/kg TCPOBOP (i.p.) in corn oil prior to treatment with saline or LPS (2 mg/kg) and livers were harvested at 16 h ($n = 5$ per group). All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in TCPOBOP/saline-treated mice was set to 1, fold change after LPS treatment was compared to the saline-treated controls. # indicates significant difference ($p < 0.05$) between saline and LPS groups.

Fig 5: Regulation of DME and transporter mRNA levels by LTA or LPS in hCAR mice pre-treated with PB. hCAR mice were treated with the universal CAR activator PB (80 mg/kg/day) for 3 days prior to i.p. administration of: (A) saline or LTA (6 mg/kg) for 4 h and (B) 16 h. (C)
hCAR mice were treated with PB 80 mg/kg/day for 3 days prior to saline or LPS (2 mg/kg) treatment for 16 h. Livers were harvested and mRNA levels were analyzed by real-time PCR as described earlier. All data are presented as ±SD and standardized for cyclophilin mRNA levels. Expression in saline-treated mice was set to 1, fold change after LTA or LPS treatment was compared to the saline-treated controls. * indicate significant difference \((p < 0.05)\) between saline and LTA or LPS groups and # indicates significant differences between saline samples of –PB and +PB groups. The experiments were repeated at least thrice.

Fig 6: (A) Regulation of DME and transporter gene expression by LTA or LPS in primary hepatocytes from CAR\(^{+/+}\) and CAR\(^{-/-}\) mice. Primary hepatocytes from CAR\(^{+/+}\) and CAR\(^{-/-}\) mice were treated with: (A) saline or LTA (50 ng/ml) for 8 h, or (B) saline or LPS (1 \(\mu\)g/ml) for 16 h. RNA was isolated, and real-time PCR was performed as described earlier. \(n = 5-6\) per group. All data are presented as ±SD and standardized for cyclophilin mRNA levels. * indicate significant difference \((p < 0.05)\) between saline and LTA/LPS of CAR\(^{+/+}\) groups and ‡ indicates significant differences \((p < 0.05)\) between saline samples of CAR\(^{+/+}\) and CAR\(^{-/-}\) groups and # indicates significant difference \((p < 0.05)\) between saline and LTA/LPS of CAR\(^{-/-}\) group. The experiments were repeated at least thrice.

Fig 7: (A) Regulation of DME and transporter gene expression by LTA in TCPOBOP pre-treated primary hepatocytes. Primary hepatocytes from CAR\(^{+/+}\) were isolated and treated with specific CAR activator TCPOBOP (250 nM) for 24h prior to treatment with: (A) saline or LTA (50 ng/ml) for 8 h, or (B) saline or LPS (1 \(\mu\)g/ml) for 16 h. RNA was isolated, and real-time PCR
was performed as described earlier. \( n = 5 \) per group. All data are presented as ±SD and standardized for cyclophilin mRNA levels. * indicate significant difference \((p < 0.05)\) between saline and LTA/LPS of –TCPOBOP (TC) groups and ‡ indicates significant differences \((p < 0.05)\) between saline samples of –TC and +TC groups and # indicates significant difference \((p < 0.05)\) between saline and LTA/LPS of + TC group. The experiments were repeated at least thrice.