dent NAS, there is evidence for an operative ESE in the susceptible exon (*25–27*). In view of our results, it is possible that rent1/hUpf1-mediated events selectively impair utilization of such vulnerable (ESE-dependent) exons.

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Fig. S1

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Modulation of Acetaminophen-Induced Hepatotoxicity by the Xenobiotic Receptor CAR

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We have identified the xenobiotic receptor CAR (constitutive androstane receptor) as a key regulator of acetaminophen metabolism and hepatotoxicity. Known CAR activators as well as high doses of acetaminophen induced expression of three acetaminophen-metabolizing enzymes in wild-type but not in CAR null mice, and the CAR null mice were resistant to acetaminophen toxicity. Inhibition of CAR activity by administration of the inverse agonist ligand androstanol 1 hour after acetaminophen treatment blocked hepatotoxicity in wild type but not in CAR null mice. These results suggest an innovative therapeutic approach for treating the adverse effects of acetaminophen and potentially other hepatotoxic agents.

Overdoses of acetaminophen (APAP; also known as 4′-hydroxyacetanilide, *N*-acetyl-*p*-aminophenol, and paracetamol) are the leading cause of hospital admission for acute liver failure in the United States (*1*). Ingestion of amounts of APAP only two to three times the maximum daily recommended dose can cause hepatotoxicity, and higher doses result in centrilobular necrosis that is potentially fatal (*2, 3*). The basis for this toxicity has been well studied. Particularly at high doses, cytochrome P-450 enzymes—especially CYP1A2, CYP2E1, and isoforms of CYP3A—convert APAP to a reactive quinone form, *N*-acetyl-*p*-benzoquinone imine (NAPQI) (*4–7*), that covalently binds to cellular macromolecules and also causes production of reactive oxygen species (*8, 9*). At subtoxic doses, NAPQI is inactivated by glutathione S-transferases (GSTs) via conjugation with reduced glutathione (GSH), but NAPQI accumulates when GSH levels are depleted. Among the numerous GST enzymes, the GSTPi isoforms are particularly effective at inactivating NAPQI (*10*). Their importance in APAP toxicity was confirmed by the unexpected demonstration that knockout mice lacking both GSTPi isoforms are relatively resistant to APAP hepatotoxicity because of a decreased rate of GSH depletion (*11*).

APAP toxicity is increased in both humans and rodents by pretreatment with various inducers of CYP gene expression, including ethanol, an inducer of CYP2E and CYP3A isoforms (*12, 13*), and phenobarbital (PB), a well-known inducer of CYP2B, CYP3A, and other isoforms (*14, 15*). Because the xenobiotic receptor CAR has recently been shown to mediate the effects of PB and other PB-like inducers (*16–19*), we examined the effect of PB and the potent CAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) on APAP toxicity in wild-type and CAR knockout mice. We treated both strains of mice with inducers or the vehicle control, followed by APAP (250 mg per kg of body weight) (*20*). Neither the inducers alone nor this dose of APAP induced hepatotoxicity, as indicated by either the serum levels of the liver enzyme alanine aminotransferase (ALT) or histologic examination, but animals treated with PB or TCPOBOP plus APAP showed elevated ALT levels and hepatic necrosis at 24 hours (Fig. 1, A and B). The CAR null mice showed no such hepatotoxicity.

Among genes associated with APAP toxicity, PB or TCPOBOP treatment modestly suppressed CYP2E1 mRNA levels but induced CYP1A2, CYP3A11, and GSTPi mRNAs in wild-type animals (Fig. 1C). We observed neither suppression of CYP2E1 nor induction of the other enzymes in CAR null mice. The strong induction of GSTPi expression by PB or TCPOBOP treatments in wild-type mice suggests that GSH depletion could contribute to the xenobiotic-induced toxicity, and wild-type mice pretreated with PB or TCPOBOP showed about a 50% decrease in hepatic GSH 2 hours after administration of APAP (Fig. 1D). To rule out the possibility that the CAR knockout animals are somehow resistant to NAPQI, we directly injected the metabolite into the livers of both wild-type and CAR null mice, and serum ALT levels increased in both by a factor of 5 to 10 (*21*). Hence, both increased NAPQI production and GSH depletion may contribute to xenobiotic-induced APAP toxicity.

To determine whether CAR null mice are

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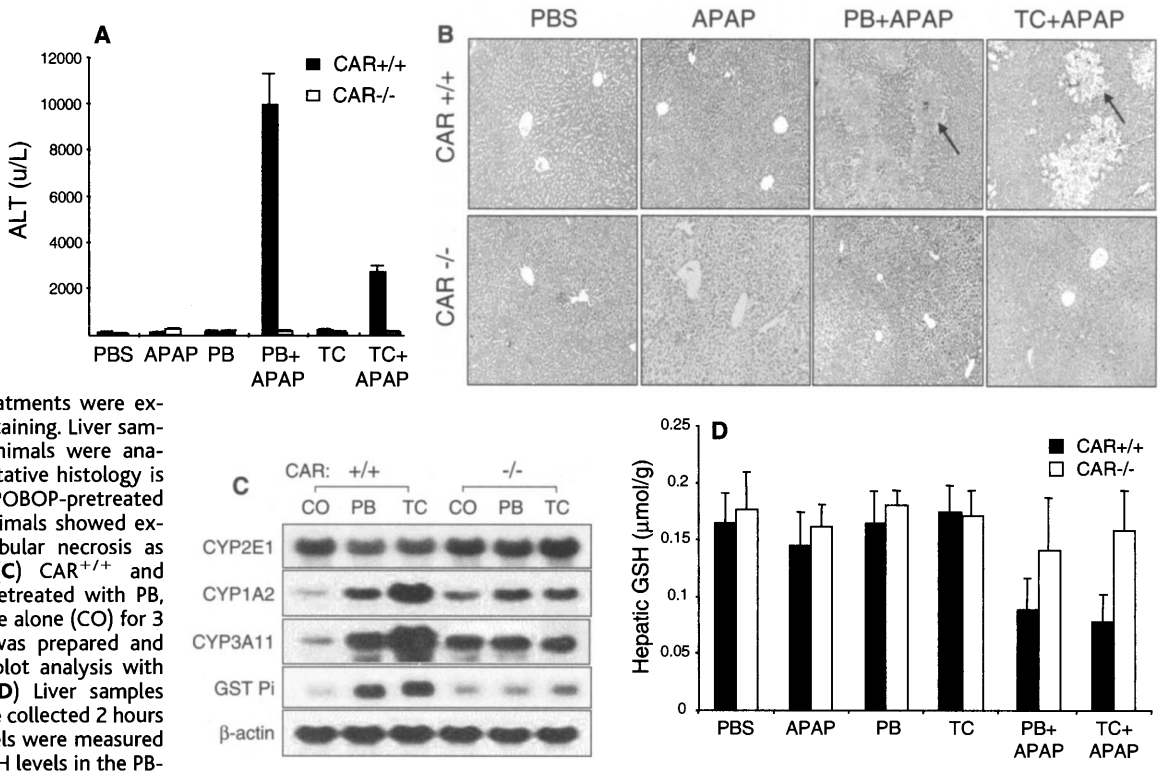

Fig. 1. CAR activation induces APAP toxicity. (**A**) $CAR^{+/+}$ or $CAR^{-/-}$ animals pretreated with PB, TCPOBOP (TC), or vehicle alone were administered a 250-mg/kg dose of APAP by intraperitoneal injection (n = 5 to 7 per treatment group). Serum was collected and ALT levels were measured after 24 hours. (**B**) Liver sections from different treatments were examined by histological staining. Liver samples from all treated animals were analyzed, but only representative histology is presented. PB- and TCPOBOP-pretreated livers from wild-type animals showed extensive hepatic centrilobular necrosis as indicated by arrows. (**C**) $CAR^{+/+}$ and $CAR^{-/-}$ animals were pretreated with PB, TCPOBOP (TC), or vehicle alone (CO) for 3 days. Total liver RNA was prepared and subjected to Northern blot analysis with the indicated probes. (**D**) Liver samples treated as indicated were collected 2 hours after APAP and GSH levels were measured (n = 5 to 7). Hepatic GSH levels in the PB- and TC-pretreated wild-type animals were significantly different from those of both the $CAR^{+/+}$ animals injected with vehicle alone (P = 0.011 and P = 0.0046, respectively) and the $CAR^{-/-}$ animals pretreated with PB and TC (P = 0.034 and P = 0.026, respectively).

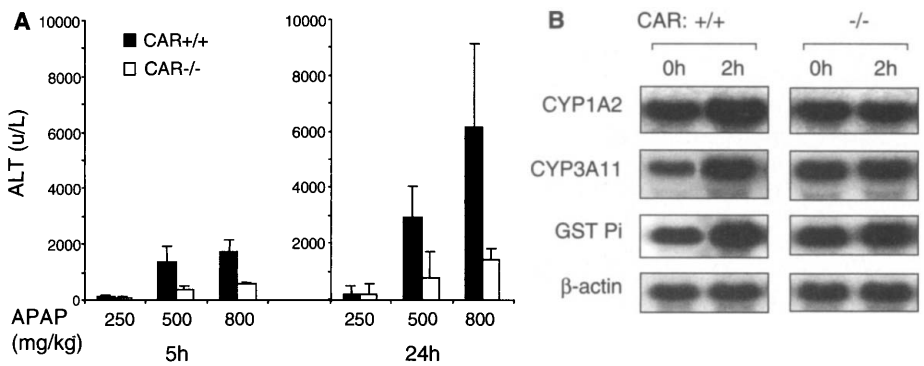

Fig. 2. $CAR^{-/-}$ mice are resistant to APAP toxicity. (**A**) $CAR^{+/+}$ and $CAR^{-/-}$ animals were given 250-, 500-, or 800-mg/kg doses of APAP. Blood samples were collected 5 or 24 hours later, and serum ALT levels were measured (n = 5 to 7). At the higher doses, $CAR^{-/-}$ animals were significantly less sensitive than wild-type animals to APAP toxicity (with APAP at 500 mg/kg, P = 0.019 for 5 hours and P = 0.026 for 24 hours; with APAP at 800 mg/kg, P = 0.016 for 5 hours and P = 0.0008 for 24 hours). (**B**) Total liver RNA was prepared from $CAR^{+/+}$ and $CAR^{-/-}$ livers treated with APAP (500 mg/kg) at 0 and 2 hours as indicated. Total RNA (10 μg) from different samples was subjected to Northern blot analysis with the indicated probes. The same blot was stripped and reprobed with β-actin as a loading control. Quantitation of mRNA levels by densitometry showed that induction of CYP1A2, CYP3A11, and GSTPi was about 2.8-, 4.4-, and 3.9-fold, respectively.

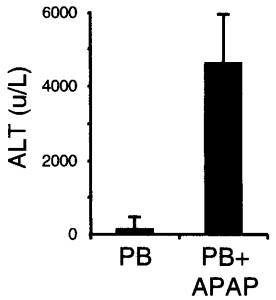

Fig. 3. Sensitivity to APAP of mice expressing human CAR. Mice expressing hCAR (n = 3) were pretreated with PB or vehicle alone for 3 days and administered a 250-mg/kg dose of APAP by intraperitoneal injection. Serum was collected and ALT levels were measured after 24 hours.

also resistant to toxic doses of APAP, we treated wild-type and knockout animals with the analgesic at 500 and 800 mg/kg. At 5 and 24 hours, the CAR knockout animals showed lower serum ALT levels than the wild-type animals (Fig. 2A). This resistance suggests that the CAR null animals may lack a xenobiotic response to the drug itself. To avoid complications associated with the extensive necrosis observed at later times, we examined CYP1A2, CYP3A11, and GSTPi expression 2 hours after administration of APAP (500 mg/kg). Even at this early time, expression of all three mRNAs was increased in wild-type but not in CAR null mice (Fig. 2B). The resistance of CAR null animals to APAP toxicity was also demonstrated by the absence of the initial stages of both hepatocellular damage and GSH depletion observed with the wild-type animals (fig. S1). Thus, loss of CAR function results in resistance to APAP toxicity that is associated with absence of the induction of APAP-metabolizing enzymes. Because neither APAP nor NAPQI functions as a CAR agonist (*21*), this activation may be similar to that of PB, which is based on induction of nuclear translocation rather than direct ligand binding (*18, 22, 23*).

The marked species differences in responses of xenobiotic receptors to various stimuli (*18, 19, 24*) raise the important question of whether the effects observed with murine CAR can also be observed with human CAR (hCAR). To test this, we generated a line of transgenic mice specifically expressing hCAR in the liver by using a construct based on the albumin promot-

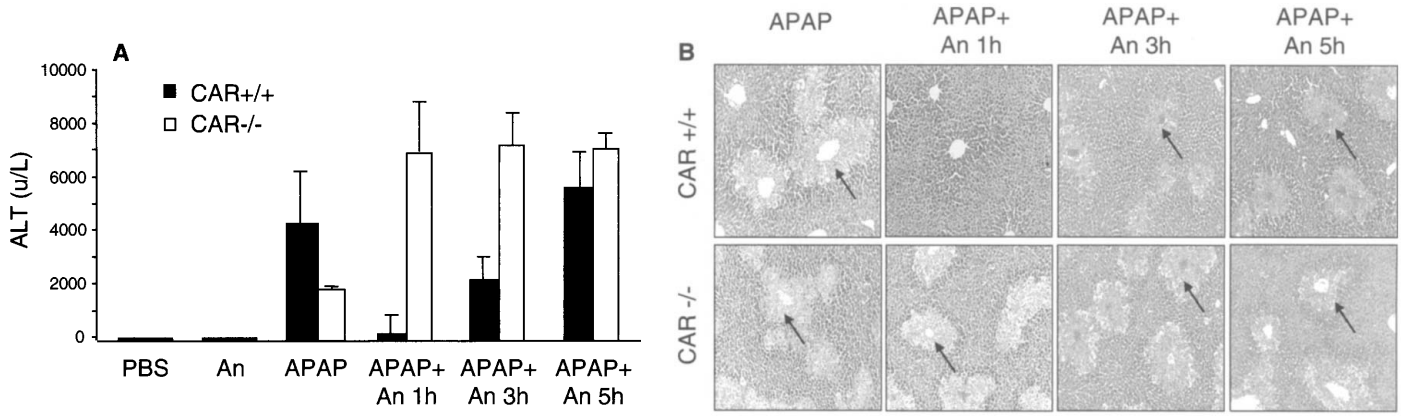

Fig. 4. Hepatoprotection by androstanol treatment. (**A**) $CAR^{+/+}$ or $CAR^{-/-}$ animals were given a 500-mg/kg dose of APAP by intraperitoneal injection, with or without an additional injection of androstanol (An; 100 mg/kg) 1, 3, or 5 hours later. Serum ALT levels were measured 24 hours later (n = 5 to 7). At 1 and 3 hours, $CAR^{+/+}$ animals showed significantly lower ALT levels than did $CAR^{-/-}$ animals (both $P < 0.0001$). (**B**) Liver sections from the same animals 24 hours after different treatments, as indicated, were stained with hematoxylin and eosin. Arrows indicate areas of hepatic necrosis.

er (fig. S2A) (*20*). We bred these hCAR-expressing animals with CAR knockout mice to produce "humanized" mice expressing only hCAR in the liver, which are analogous to previously described mice expressing only the human xenobiotic receptor PXR (pregnane X receptor) (*25*). Treatment of mice expressing hCAR with the general CAR activator PB induced expression of CYP1A2 and CYP3A11 mRNAs (*21*) and resulted in increased sensitivity to APAP (Fig. 3). Treatment of these mice with APAP at 500 mg/kg also increased expression of CYP1A2, CYP3A11, and GSTPi, which indicates that hCAR is activated by APAP (fig. S2B).

Transactivation of target genes by mouse, but not human, CAR can be blocked by the inverse agonist androstanol (*26*). Androstanol treatment not only prevents induction but also decreases basal expression of CAR target genes, including *CYP3A11* (*27*). Preliminary results demonstrated that androstanol pretreatment decreased APAP toxicity in wild-type mice. Because of the potential therapeutic implications, we were interested in whether androstanol administration after APAP treatment would have a similar effect. We gave single injections of androstanol at various times after administration of APAP (500 mg/kg) to wild-type or CAR null mice. Wild-type mice treated with the inverse agonist 1 hour after APAP administration showed a nearly complete absence of hepatotoxicity (Fig. 4). This hepatoprotective effect is mediated by CAR, because androstanol treatment did not block toxicity in identically treated CAR knockout animals (Fig. 4). Even 3 hours after APAP treatment, when modest levels of hepatic necrosis are already evident in APAP-treated mice (fig. S1A), androstanol treatment of the wild-type mice resulted in somewhat lower serum ALT levels relative to untreated wild-type animals. The protective effect of androstanol at 1 and 3 hours was very similar to that of the antioxidant *N*-acetylcysteine, which is used therapeutically to treat APAP overdose (fig. S3).

The serum ALT levels in the $CAR^{-/-}$ animals treated with both androstanol and APAP were higher than those of the CAR null mice treated with APAP alone (Fig. 4A). Because androstanol reportedly is a weak PXR agonist (*24*), this increase may be due to an activation of PXR that becomes evident in the absence of functional CAR. Treatment of wild-type mice with the strong PXR agonist 5-pregnen-3β-ol-20-one-16α-carbonitrile resulted in an increase in sensitivity to APAP comparable to that observed with TCPOBOP pretreatment (*21*). Relative to the wild-type control, androstanol treatment had no protective effect when administered 5 hours after APAP treatment.

We conclude that CAR is a central mediator of APAP toxicity in mice and potentially also in humans. CAR apparently is not involved in the toxicity associated with ethanol-dependent induction of CYP2E1 and other targets (*12, 13*), because CAR activation modestly decreased CYP2E1 mRNA levels. However, activation of either mouse or human CAR by appropriate inducers, including APAP itself, increased production of APAP-metabolizing enzymes and increased toxicity. Fatal outcomes have been reported for the combination of PB and APAP in humans (*28, 29*). Current therapeutic approaches to APAP toxicity are primarily based on treatments with reducing agents to replenish GSH levels (*30, 31*). The results described here suggest a new strategy based on CAR inverse agonists. A potential problem with this strategy is that the androstane inverse agonists that potently inhibit murine CAR have only a very limited effect on the human receptor (*26*). However, it is an exciting possibility that the identification of potent and specific new inverse agonists for human CAR may provide a clinically useful means to treat toxicity of APAP and potentially other hepatotoxic agents.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S3

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