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Regulation of the human cathepsin E gene by the constitutive androstane receptor

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Abstract

Cathepsin E (CTSE) is an aspartic protease that has been linked to antigen processing and innate immunity. Elevated levels of CTSE expression have also been associated with several forms of cancer, including carcinomas exhibiting highly invasive character. In this study, we performed DNA microarray experiments, together with quantitative reverse transcriptase PCR analyses and enzymatic activity determinations to identify human CTSE as a novel target gene for regulation by the constitutive androstane receptor (CAR), a nuclear receptor activated by the liver tumor promoting agent, phenobarbital. In particular, two motifs within the 5′-flanking region of the human CTSE gene were identified as direct sites of interaction with CAR/RXRα heterodimers, a direct repeat-3 site at position −766 and a direct repeat-4 site at position −1407. Thus, these studies demonstrate CAR-mediated regulation of CTSE within primary hepatocyte cultures from several individual donors and suggest that elevated CTSE activity may play a functional role in the etiology of hepatocarcinogenesis.

Keywords

Cathepsin E; Constitutive androstane receptor; Hepatocytes; Primary hepatocytes; Liver; Human; Hepatocytes

Cathepsin E (CTSE)[1] is an intracellular aspartic protease that hydrolyzes various biologically active peptides [1]. Unlike related proteases, CTSE is localized outside of the lysosomal system and likely functions in extralysosomal proteolysis [2]. Although its exact biological functions remain elusive, several physiological roles of CTSE have been reported, including roles in immune defense and antigen processing [3-5]. In this respect, CTSE-deficient mice exhibit markedly increased susceptibility to both gram positive and gram negative bacterial infection, likely due to impaired regulation of bacterial elimination [5].

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[1]Abbreviations used: CTSE, cathepsin E; PB, phenobarbital; CAR, constitutive androstane receptor; DMSO, dimethyl sulfoxide; RFUs, relative fluorescent units.

CTSE deficiency also is associated with a novel form of lysosomal storage disorder in macrophages [6]. A related protease, cathepsin D, has been implicated in the degradation of extracellular matrix proteins [7]. CTSE has been similarly reported to be secreted extracellularly [8].

An apparent hallmark feature of CTSE is its association with several types of human cancers. Gastric carcinomas, pancreatic tumors, colon carcinomas and hepatocellular carcinomas all exhibit elevated levels of CTSE [9-13]. In this respect, immunocytochemical analyses indicated that CTSE was most intensely localized at the leading edge of invasive gastric carcinomas [11]. Gene expression level comparisons between metastatic and non-metastatic hepatocellular carcinomas demonstrated increased CTSE expression associated with metastatic tumors [10].

Cell selective expression patterns of CTSE likely govern its specific functions. The tissue distribution of CTSE varies according to cell type and species [1]. Relatively high levels of expression occur in cells of the gastric system, Clara cells of the lung, erythrocytes and inflammatory cells, with lower expression noted in human spleen [11,14,15]. Saku et al. [2] demonstrated expression of CTSE localized within the bile canaliculi of the rat liver as well as microvilli of hepatic cells.

Given the apparent association of enhanced CTSE expression with hepatocellular carcinoma, we hypothesize that liver tumor promoting agents may function to enhance CTSE expression levels in liver and that increased proteolytic activity may underlie certain mechanistic aspects of the tumor promotion process. In the studies reported here, we identify CTSE expression in human hepatoctyes. In particular, microarray expression profiling analyses indicated that exposures to the classical liver tumor promoter, phenobarbital (PB), resulted in increased CTSE transcript levels within primary hepatocyte cultures from several individual donors, a result that was confirmed by quantitative RT-PCR experiments. Further, we demonstrate that the constitutive androstane receptor (CAR) acts as a transcriptional regulator of the human CTSE gene and that these events lead to PB-inducible increases in functional hepatic CTSE enzymatic activity.

Materials and methods

Chemicals

Phenobarbital sodium salt was obtained from UWMC Drug Services (Seattle, WA). Clotrimazole, meclizine and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). CITCO was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Cell culture

COS-1 cells were maintained and transfected in Dulbecco’s modified Eagle medium with 10% FBS, 2 mM L-glutamine, 10 mM Hepes, 0.15% sodium bicarbonate, 50 U/ml penicillin G and 50 μg/ml streptomycin. Primary human hepatocyte cultures were maintained as described previously [16]. Selected cultures were treated with 500 μM PB or 5 μM CIT- CO,

on day 4 of culture. All cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA).

RNA isolation/cDNA preparation and RT-PCR/RT-PCR data and microarray analysis

All methods were performed as described previously [16].

hCTSE promoter analyses

Sequence information from the 5′ end of the hCTSE locus was obtained by a query of the Ensembl Human Genome Browser. hCTSE promoter deletions were created with PCR, using XhoI-hCTSE forward oligonucleotide primers, reverse Rev.CTSEprom primer 5′-TGTGAG TCCGACCAGCAGCTCTCC-3′, and the BAC clone RP11-38J22 (BACPAC Resources, Children's Hospital Oakland, Oakland, CA, USA) as template for Accupol polymerase (Genechoice, Frederick, MD). All PCRs were supplemented with 1 M Betaine (USB Corp., Cleveland, OH). PCR products were digested with XhoI and subcloned into XhoI/EcoRV linearized pGL4 (Promega, Madison, WI), containing a thymidine kinase enhancer. pGL4-TK was constructed as described previously [17,18]. −5308CTSE-pGL4TK and −4118CTSE-pGL4TK constructed by amplification with the XhoI-hCTSE were forward primer and a reverse primer, 5′-GCCCATTCTGAAAATCCTAGGGCCC-3′, containing an internal AvrII site. PCR products were digested with XhoI and AvrII and subcloned into XhoI/AvrII linearized −3115CTSE-pGL4TK. Constructs were verified by sequence analysis. Other plasmids used in this study include: CMV2, CMV2-CAR, pcDNA3.1, pcDNA3.1-RXRα, pRL-CMV, 3XFlag, and 3XFlag-CAR. Prior to transfection, plasmids were purified using the Qiagen Plasmid Maxi Kit (Qiagen Inc., Valencia, CA).

Luciferase assays

All transfections were performed in COS-1 cells in a 48-well format. On day one, cells were plated to approximately 50,000 cells per well. While the cells were attaching, DNA transfection mixtures were prepared using Fugene6 transfection reagent (Roche, Indianapolis, IN) in a 3:1 (reagent:DNA) ratio. For assays involving CTSE promoter reporters, 25 ng of CMV2 or CMV2-CAR expression plasmid, 25 ng pcDNA3.1 or 3.1-RXRα expression plasmid, 100 ng luciferase reporter and 10 ng of pRL-CMV (Promega, Madison, WI.) were used. Selected cultures were treated with 500 μM PB, 10 lM CITCO, 10 μM meclizine, 10 μM clotrimazole or DMSO after 24 h. On day 3, cells were washed with PBS and luciferase assays were conducted using the Dual-Luciferase TM Reporter Assay System (Promega) and quantified with a Veritas Microplate Luminometer TM (Turner Biosystems, Sunnyvale, CA.). Luciferase assay and stop and glow reagents were diluted with 1 × TBS (Tris buffer saline, pH 8.0) to a 0.5× final concentration. All other aspects of the assay were performed in accordance to the manufacturer's protocol. Dilution of luciferase reagent had no effect on normalized luciferase values.

EMSAs

The entire 5.3 kb upstream promoter region of hCTSE was analyzed using the web based service, NHRScan (http://mordor.cgb.ki.se/cgi-bin/NHR-scan/nhr_scan.cgi), to identify potential nuclear receptor binding motifs [19]. The results were used to target construction

of specific oligomers for EMSAs. 6 μg of 3XFlag or 3XFlag-CAR expression plasmid, 6 μg pcDNA3.1 or 3.1-RXRα expression plasmid were transfected into 100 mm culture dishes of freshly plated COS-1 cells. After 48 h, nuclear extracts were harvested using NE-PER® kit (Pierce Biotechnology, Rockford, IL). Nuclear extracts were quantified by UV absorbance and a Bio-Rad protein assay (Bio-Rad, Hercules, CA). EMSAs were performed using ^{32}P-labeled, double stranded oligomers corresponding to putative CAR/RXRα binding sites (Integrated DNA Technologies, Coralville, IA). Labeled probes were incubated with 5 μg nuclear protein with α-Flag antibody (Sigma, St. Louis, MO) or 200× cold probe at room temperature for 20 m. After addition of loading buffer, samples were electrophoresed on 5% TBE pre-case gels (Bio-Rad, Hercules, CA) in 0.5× TBE buffer for 1 h at 120 volts. Gels were then dried and visualized by autoradiography.

Fluorometric assay

Five day cultures of primary human hepatocytes, control or treated for 24 h with 500 μM PB or 5 μM CITCO, were lysed in 150 μL of CHAPS lysis buffer (Boston Bioproducts, Worcester, MA). In a 96-well plate, 20 μL of sample was combined with 70 μL 50 mM sodium acetate buffer, pH 4.0 and 10 μL of 200 μM substrate solution (Peptides International, Louisville, KY). The plate was incubated at 37 °C for 15 m and then scanned every 15 m Fluorcount (Perkin-Elmer, Wellseley, MA) at 37 °C. Results were normalized to blanks (substrate only) and expressed as relative fluorescent units.

Results

Microarray and RT-PCR

RNA was collected from day 6 cultures of primary human hepatocytes that were treated with PB, a potent inducer of CAR, for 24 h and processed for microarray hybridization. Parallel samples were subjected to quantitative RT-PCR (Fig. 1b). Results from microarray data analysis demonstrated that hepatocytes from 8 of the 10 individuals assayed exhibited PB-inducible increases in CTSE transcript expression (Fig. 1a). The maximal response to PB treatment was seen in Donor D, with almost a 20-fold up-regulation of CTSE; whereas the other PB responsive individuals exhibited 2- to 3-fold increases in CTSE transcript in response to PB. CTSE transcript levels were down regulated by PB in 2 donors, with Donor B and Donor H exhibiting 2- and 10-fold decreased expression levels, respectively (Fig. 1a). RT-PCR results confirmed these trends, with 7 of 10 individuals exhibiting an overall increase in CTSE transcript levels. Donor D demonstrated a ~3-fold induction of CTSE, and Donor H a 2.5-fold reduction in transcript level (Fig. 1b).

Human CTSE (hCTSE) promoter analysis

Since the hCTSE gene appeared PB-inducible in most individuals, we hypothesized that the gene was likely a target for CAR regulation. We assessed ~5.3 kb of the hCTSE 5′ upstream promoter region with NHRScan [19] to reveal locations of putative CAR/RXRα heterodimer binding motifs. The output indicated that eight potential motifs existed, as follows: a DR-3 at −766, a DR1 at 1161, an IR-1 at −1379, a DR-4 at −1407, a DR-1 at −1672, an ER-6 at −2384, a DR-4 at −4031, and an IR-1 at −5205 (Fig. 2a).

A series of six sequential deletion plasmids were constructed containing 818, 1218, 1768, 3115, 4118, and 5308 bp of sequence, respectively, immediately upstream of the CTSE start codon, with the respective hCTSE promoter regions driving expression of the luciferase reporter gene. These constructs were used in conjunction with expression plasmids for CAR and RXRα in transient transfection assays of COS-1 cells. All of the hCTSE promoter reporter constructs exhibited transactivation activity with CAR/RXRα co-transfection, but not with CAR or RXRα when transfected individually. Overexpression of CAR/RXRα with −5308, −4118, and −3115CTSE-pGL4TK induced activity ~30× that of vector alone, with the −1768 construct exhibiting less activity, only 10× the activity of vector alone. The last two regions, i.e., −818 and −1218, of the CTSE promoter showed the same activity, ~ 5-fold over the vector control, indicating that a single shared site was likely contributing to the activation. Taken together, the results indicated that one or more binding/activation sites may exist between −1 to −818, −1218 to −1768, and −1768 to −3115 (Fig. 2b).

EMSAs

To determine more definitely if CAR/RXRα heterodimers directly interact with any of the putative nuclear receptor binding sites, EMSAs were performed with ^{32}P radiolabeled oligonucleotides, corresponding to NHRScan output sequences within the fragments whose activity profiles were assayed in Fig. 2. In addition to the radiolabeled probe, nuclear protein interactions were also incubated with an anti-Flag antibody or unlabeled probe prior to gel separation. Fig. 3 illustrates that CAR/RXRα heterodimers directly bind to the DR-3 site at −766 and the DR-4 site at −1407. Both probes demonstrated a shift upon addition of nuclear extract containing overexpressed CAR and RXRα (Fig. 3, lower arrows). No shift is apparent in empty vector controls. Also, those samples that were incubated with the Flag antibody (Fig. 3, lanes 4 and 8) exhibited a supershift (Fig. 3, upper arrows), confirming the identity of the protein as the Flag-tagged CAR. The resulting bands were effectively reduced by addition of 100× excess cold competitor probe.

CTSE enzyme activity assay

PB is a classical activator of the CAR pathway [20,21]. To examine whether the binding and transactivation of hCTSE promoter sequences by CAR resulted in an increase in actual CTSE activity, a fluorometric assay was performed. The results of the CTSE activity assays demonstrated that primary cultures of human hepatocytes treated with PB or CITCO possessed significantly higher levels of fluorescence at all incubation time points, indicating increased CTSE activity and corresponding cleavage of the peptide substrate. CITCO treatment yielded higher levels of enzymatic activity than treatment with PB (Fig. 4).

Activation of hCTSE by CAR splice variants

The human CAR gene produces several splice variants that exhibit unique functional characteristics and are expressed in liver tissues simultaneously with the reference form of CAR, CAR1 [22-25]. In order to investigate whether select CAR splice variants activate the hCTSE promoter, the largest promoter construct was used to examine potential hCTSE transcriptional modulation by the prominent hCAR variants, CAR2 and CAR3 [22]. In these studies, expression plasmids for CAR1, CAR2, CAR3, and RXRα were used to transiently transfect COS-1 cells and assessed for their respective transactivation potential with the

5308 bp hCTSE promoter construct. Luciferase activity analysis demonstrated that both CAR2 and CAR3 activated transcription of the hCTSE promoter, though not to the extent of CAR1 (Fig. 5). CAR2 demonstrated constitutive activational activity with the hCTSE promoter, displaying ~40% of the activation potential of CAR1. The ability of CAR3, a ligand activated form of CAR, only modestly activated transcription of the hCTSE promoter fragment, at ~15% of the activity demonstrated by CAR1. Treatment with a CAR antagonist, clotrimazole [26-28], repressed activity of both CAR1 and CAR2. Exposures to a CAR1 inverse agonist, meclizine [29], resulted in modest repression of CAR1 activity, and no apparent alteration of CAR2 activity.

Discussion

CTSE is an aspartic protease that has been linked to antigen processing and innate immunity. The tissue distribution of CTSE expression appears highest in the stomach, lungs and cells of the immune system [11,14,15]. There are also reports of lower levels of expression in the rat liver, particularly in the bile canaliculi and microvilli [2,14]. Previous studies of CTSE transcriptional regulation indicated that CTSE was positively regulated by several cell-type specific transcription factors, including GATA-1, erythroid specific, PU.1, pancreatic, and Isl1, hematopoietic [14]. A ubiquitously expressed transcription factor, YY1, was reported as a negative regulator of CTSE promoter activity [14]. Later, other studies revealed that the transcription factor CIITA, specific to dendritic and B cells, also negatively regulates the expression of CTSE [30]. The results presented in this study demonstrate that human CTSE is a novel gene target for CAR and that CTSE is transcriptionally regulated by PB and the CAR ligand, CITCO.

Hepatocellular carcinoma is the most prevalent type of liver cancer, comprising ~90% of liver tumors occurring in humans [31]. In rodent models, PB is used classically as a promoting agent for hepatocellular carcinoma, following administration of genotoxic carcinogens such as dimethlnitrosamine [32]. Although the exact mechanisms underlying the tumor promotion process remain clouded, studies using transgenic mice disrupted for the CAR allele have shown convincingly that these mice are completely resistant to hepatocellular carcinoma following administration of standard 2-stage initiation-promotion regimens [33]. Thus, these studies implicate CAR activation as a critical pathway in liver cancer development.

In these respects, CAR is expressed primarily in the liver and its role as an important regulator of genes involved in xenobiotic metabolism is well-established [34]. Typically, at least in the *in vivo* context, CAR is localized preferentially in the cytoplasm and translocates to the nucleus upon receptor activation by CAR selective substances. Once nuclear, CAR is free to heterodimerize with RXRα and activate a cadre of genes possessing specific nuclear hormone receptor response elements [20,21,35]. Previously unrecognized as a CAR-responsive gene, here we show that hCAR/RXRα heterodimers are capable of transactivating hCTSE promoter sequences in transfected mammalian cells. In this context, a plasmid containing 3 kb of upstream sequence yielded ~30× more activity in the presence of hCAR/RXRα than vector alone. NHRScan analyses of the human CTSE promoter region identified six putative binding motifs. In our EMSA studies, two of these sites were

identified as direct sites of interaction with CAR/RXRα heterodimers, a DR-3 site at −766 and the DR-4 site at −1407 (Fig. 3).

The up-regulation of CTSE mRNA expression by CAR also resulted in altered functional activity. When primary human hepatocytes, either untreated or treated with known CAR activators, were assayed for CTSE enzymatic levels, the results demonstrated that hepatocytes treated with PB or CITCO exhibited significantly higher CTSE enzymatic activity than untreated hepatocytes (Fig. 4), with CTSE activities significantly increased at all measured time intervals (15, 30, 45, and 60 m; $p \leq 0.01$ or $p \leq 0.05$). The largest cathepsin E promoter region tested was also activated by two previously characterized hCAR splice variants, CAR2 and CAR3 (Fig. 5). Though perhaps it is not surprising that splice variants of hCAR exert similar effects on target genes, it is interesting that the magnitude of these effects varies greatly on the CTSE promoter, supporting a view that specific CAR splice variants may exhibit differential activities on various gene promoters.

Although the biological role of CTSE is as yet unclear, CTSE function has been linked to several processes such as antigen presentation, immunity and defense, for example, CTSE mouse knockout models exhibit increased susceptibilities to bacterial infections [4,5]. In addition to its likely role in immunity, elevated levels of CTSE have now been identified in several types of cancer, including gastric carcinomas, pancreatic tumors, colon carcinomas, and hepatocellular carcinomas [9-13]. It is noteworthy that CTSE is most intensely localized at the leading edge of invasive gastric carcinomas [11], and exhibits enhanced expression specifically in hepatocellular carcinomas of highest metastatic potential [10]. From these observations, one may predict that CTSE has a functional role in the metastatic process. Although controversial, there is evidence for CTSE release from the cell into the extracellular milieu [8]. A closely related protease, cathepsin D, is secreted extracellularly and has been implicated as a marker of mammary cancer invasiveness [7,36,37]. We suggest that CTSE may share overlapping function with that of cathepsin D. In these respects, PB is a well-known promoter of hepatocellular carcinoma [38,39]. We speculate that PB may act mechanistically to promote tumor formation, in part through a CAR-mediated activation of CTSE which may function in the proteolytic digestion of the extracellular matrix, thereby facilitating the invasion of tumor cells within their local microenvironment. Further investigations will be required to test these ideas.

Acknowledgments

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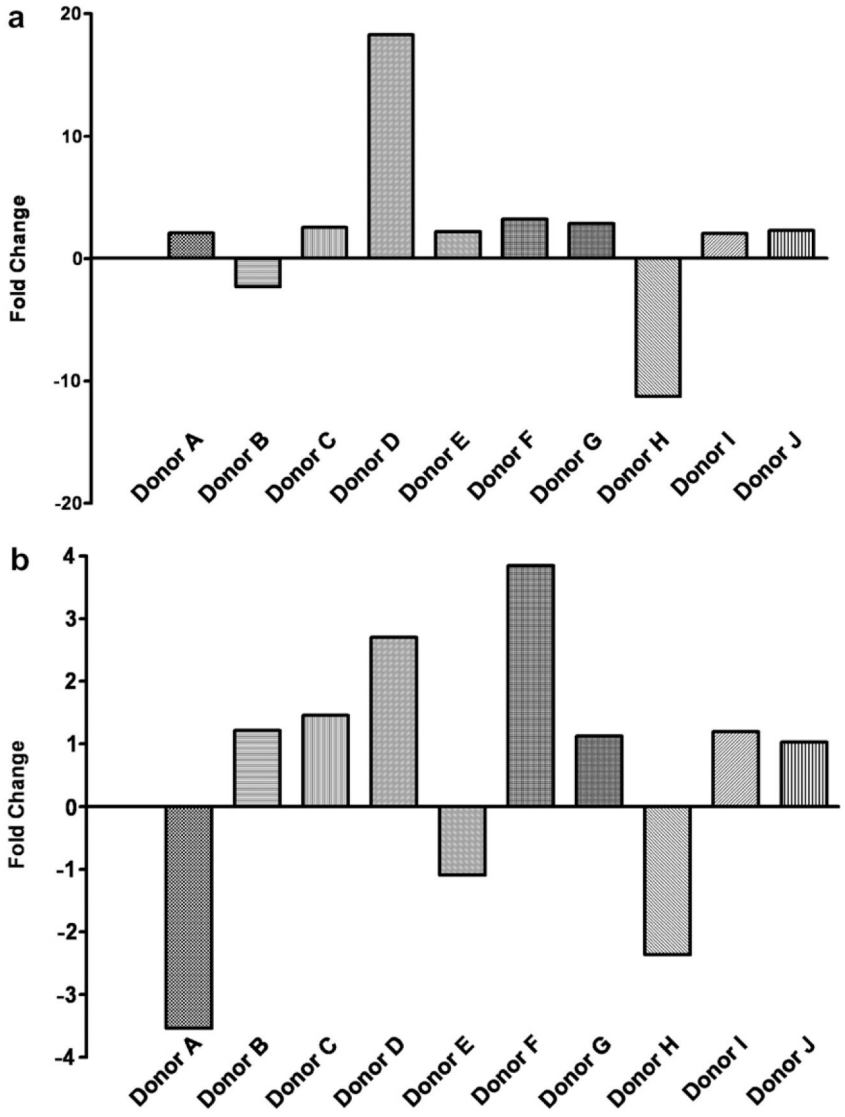

Fig. 1.

Cathepsin E is induced by phenobarbital treatment in primary human hepatocyte cultures. RNA was purified from cultures of primary human hepatocytes, control and those treated with 500 M phenobarbital, and subjected to both microarray (a) and quantitative RT-PCR analysis (b). Results are expressed as fold-change relative to control cultures.

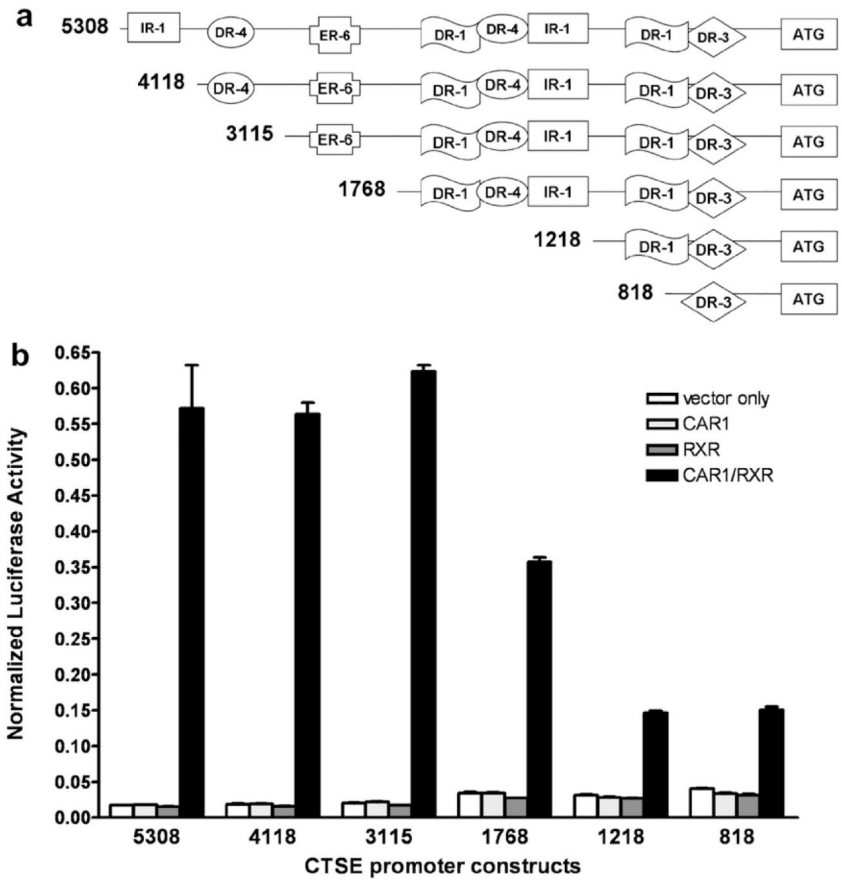

Fig. 2.

CAR/RXRα heterodimers activate transcription of cathepsin E promoter constructs. The sequence of the cathepsin E promoter was scanned for nuclear receptor binding sites by NHRScan (a). COS-1 cells were transiently co-transfected with CAR and RXRα, along with reporter constructs containing regions of the cathepsin E promoter, and assayed for luciferase reporter activity (b).

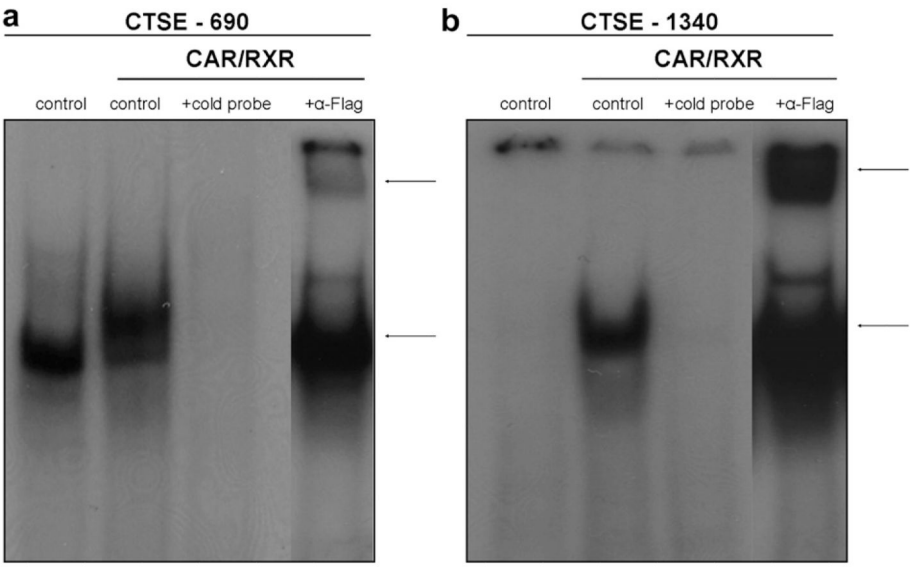

Fig. 3.

CAR/RXRα heterodimers bind to cathepsin E promoter sequences. COS-1 cells were transiently co-transfected with Flag-tagged CAR and RXRα and nuclear extracts were harvested at 48 h. Extracts were incubated with radiolabled oligonucleotides corresponding to potential binding sites in the cathepsin E promoter, together with 100× excess cold probe, or 1 μg Flag antibody. Complexes were resolved by gel electrophoresis and visualized by autoradiography. Arrows indicate shifted and super-shifted complexes.

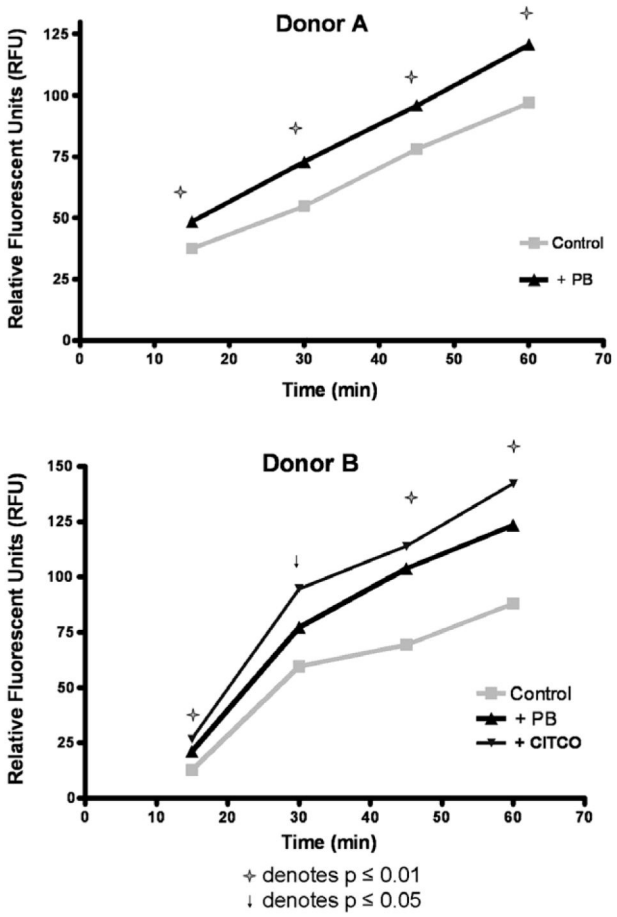

Fig. 4.

Phenobarbital and CITCO treatment of human hepatocytes results in increased cathepsin E enzyme activity. Cultures of primary human hepatocytes, control and those treated with PB or CITCO, were harvested in 150 μL CHAPS lysis buffer. Lysates were analyzed for enzymatic activity using a fluorescently-tagged substrate. Results are provided in relative fluorescent units (RFUs) versus time (m).

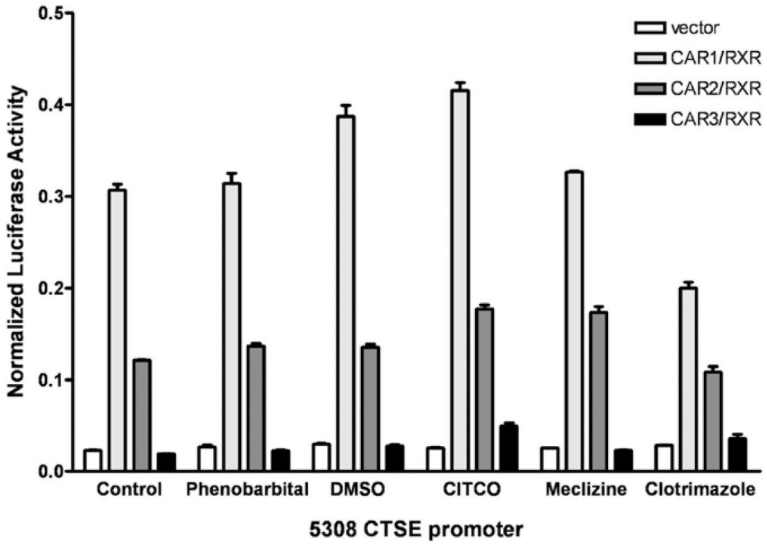

Fig. 5.

CAR splice variants activate transcription of cathepsin E promoter. COS-1 cells transiently transfected with CAR1, CAR2, or CAR3 and RXRα along with the 5308 cathepsin E reporter construct were treated with various chemicals and subsequently assayed for luciferase activity.