

Interleukin-6 Alters the Cellular Responsiveness to Clopidogrel, Irinotecan, and Oseltamivir by Suppressing the Expression of Carboxylesterases HCE1 and HCE2

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ABSTRACT

Carboxylesterases constitute a class of enzymes that play important roles in the hydrolytic metabolism of drugs and other xenobiotics. Patients with liver conditions such as cirrhosis show increased secretion of proinflammatory cytokines [e.g., interleukin-6 (IL-6)] and decreased capacity of hydrolysis. In this study, we provide a molecular explanation linking cytokine secretion directly to the decreased capacity of hydrolytic biotransformation. In both primary hepatocytes and HepG2 cells, treatment with IL-6 decreased the expression of human carboxyl-esterases HCE1 and HCE2 by as much as 60%. The decreased expression occurred at both mRNA and protein levels, and it was confirmed by enzymatic assay. In cotransfection experiments, both HCE1 and HCE2 promoters were significantly repressed, and the repression was comparable with the decrease in HCE1 and HCE2 mRNA, suggesting that

transrepression is responsible for the suppressed expression. In addition, pretreatment with IL-6 altered the cellular responsiveness in an opposite manner of overexpression of HCE1 and HCE2 toward various ester therapeutic agents (e.g., clopidogrel). Transfection of HCE1, for example, decreased the cytotoxicity induced by antithrombotic agent clopidogrel, whereas pretreatment with IL-6 increased the cytotoxicity. Such a reversal was observed with other ester drugs, including anticancer agent irinotecan and anti-influenza agent oseltamivir. The altered cellular responsiveness was observed when drugs were assayed at sub- and low-micromolar concentrations, suggesting that suppressed expression of carboxylesterases by IL-6 has profound pharmacological consequences, particularly with those that are hydrolyzed in an isoform-specific manner.

The lipophilic nature of drugs favors absorption and subsequent distribution to their target tissues (Parkinson, 2001). Metabolism of drugs, conversely, leads to increased polarity of drugs, and it favors excretion into urine and feces. The liver is the richest source of drug-metabolizing enzymes in terms of abundance and diversity, thereby playing a determinant role in drug metabolism (Parkinson, 2001). Although many factors may alter the hepatic capacity of drug metabolism, regulated expression of drug-metabolizing enzymes contributes the most to the alteration (Parkinson, 2001; Poso and Honkakoski, 2006). Transactivation by nuclear receptors such as the pregnane X receptor is largely

responsible for the increased expression of these genes (Poso and Honkakoski, 2006). In contrast, the mechanisms of the down-regulation largely remain unclear. Cytokines such as tumor necrosis factor- α and interleukin-6 (IL-6) have been shown to down-regulate the expression of a variety of drug-metabolizing enzymes (Frye et al., 2002; Prandota, 2005; Charles et al., 2006). The most investigated mechanism related to the down-regulation is on the expression of the cytochrome P450 (P450) system. In addition to drug-metabolizing enzymes, many cytokines decrease the expression of drug transporters as well as nuclear receptors that support the transactivation of genes encoding drug-metabolizing enzymes or transporters (Pascucci et al., 2000; Blokzijl et al., 2007).

Carboxylesterases constitute a class of enzymes that hydrolyze drugs containing such functional groups as carboxylic acid ester, amide, and thioester (Satoh and

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ABBREVIATIONS: IL-6, interleukin-6; P450, cytochrome P450; HCE, human carboxylesterase; LPS, lipopolysaccharide; DRB, 5,6-dichlororibosidyl-benzi-midazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; OD, optical density; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

Hosokawa, 2006). The liver expresses two major carboxylesterases, including human carboxyl-esterases HCE1 and HCE2, whereas the gastrointestinal tract expresses predominantly HCE2 (Schwer et al., 1997; Satoh and Hosokawa, 2006). In addition to the difference in tissue distribution, these two enzymes differ markedly in the hydrolysis of certain drugs. For example, HCE1 but not HCE2 rapidly hydrolyzes antithrombotic agent clopidogrel and anti-influenza agent oseltamivir (Shi et al., 2006; Tang et al., 2006). In contrast, HCE2 but not HCE1 rapidly hydrolyzes anticancer agent irinotecan (Wu et al., 2002). In the presence of ethyl alcohol, HCE1 catalyzes transesterification, which accounts for the formation of ethyl clopidogrel (Tang et al., 2006). Based on the Human Genome sequence, HCE1 has two closely related genes, namely, HCE1A1 (AB119997) and HCE1A2 (AB119998). These two forms differ by four amino acids in the signaling peptide (Tanimoto et al., 2007). It is noteworthy that the expression of HCE1A2 but not HCE1A1 mRNA is correlated better with the sensitivity to anticancer drug irinotecan, although the precise mechanism for such a phenomenon remains to be determined (Tanimoto et al., 2007). In addition, several sequences encoding HCE1 with one or more amino acid substitutions have been deposited into the GenBank, but they probably represent polymorphic variants of this carboxylesterase.

Hydrolysis of many drugs is reduced in liver diseases such as hepatitis and cirrhosis (Thiollet et al., 1992; Gross et al., 1993). In these conditions, the production of various cytokines is markedly increased (Frye et al., 2002; Zhang et al., 2002; Eriksson et al., 2004; Prandota, 2005). For example, the hydrolysis of perindopril, a nonsulphydryl angiotensin-converting enzyme inhibitor, is decreased by as much as 50% in patients with cirrhosis (Thiollet et al., 1992). Likewise, the hydrolysis of cilazapril, another antihypertensive, is decreased by 45% in hepatitis patients (Gross et al., 1993). In mice, treatment with lipopolysaccharide (LPS) causes a 70% decrease in the serum level of a carboxylesterase (Wait et al., 2005). LPS is a potent immune stimulant, and injection of LPS in rats and mice induces septic symptoms accompanied by excessive production of certain cytokines such as tumor necrosis factor- α and IL-6 (Villa et al., 1995; Remick et al., 2000). These findings suggest that cytokines down-regulate the expression of carboxyl-esterases as well.

In this study, we have demonstrated in both primary hepatocytes and HepG2 cells that treatment with proinflammatory cytokine IL-6 decreased the expression of HCE1 and HCE2. The decreased expression occurred at both mRNA and protein levels, and it was confirmed by enzymatic assay. Both HCE1 and HCE2 promoters were significantly repressed by IL-6, and the repression was comparable with the decrease in HCE1 and HCE2 mRNA. In addition, pretreatment with IL-6 altered the cellular responsiveness in an opposite manner of overexpression of HCE1 and HCE2 toward various ester therapeutic agents, including clopidogrel, irinotecan, and oseltamivir. The altered cellular responsiveness occurred at sub- and low-micromolar concentrations, suggesting that suppressed expression of carboxylesterases by IL-6 has profound pharmacological consequences.

Materials and Methods

Chemicals and Supplies. IL-6 was purchased from R&D Systems (Minneapolis, MN). Actinomycin D, 5,6-dichlororibosylbenzimidazole (DRB), irinotecan, *p*-nitrophenylacetate, Hanks' balanced salt solution, and Williams' E medium were purchased from Sigma-Aldrich (St. Louis, MO). Clopidogrel bisulfate was purchased from ChemPacific (Baltimore, MD). Oseltamivir was purchased from Toronto Research Chemicals (North York, ON, Canada). Eagle's minimal essential medium, high-fidelity Platinum TaqDNA polymerase, and insulin-transferrin-selenium G supplement were purchased from Invitrogen (Carlsbad, CA). Dual-Luciferase reporter assay system was from Promega (Madison, WI). Fetal bovine sera were from Hyclone Laboratories (Logan, UT). The antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Abcam (Cambridge, UK). The goat anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce Chemical (Rockford, IL). Nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). Unless otherwise specified, all other reagents were purchased from Fisher Scientific (Fair Lawn, NJ).

Culture and Treatment of Human Primary Hepatocyte and HepG2 Cell Line. Plated human primary hepatocytes (in six-well plates) were obtained from the Liver Tissues Procurement and Distribution System (University of Minnesota, Minneapolis, MN) or the commercial source CellzDirect (Pittsboro, NC). In total, four donors were included: two males (21 and 48 years old) and two females (35 and 72 years old). Among the donors, three were white and one was African American (the 21-year man). None of them was a smoker. Upon arrival, media were replaced with rich Williams' E medium containing insulin-transferrin-selenium supplement and 100 U/ml penicillin/10 μ g/ml streptomycin (Yang and Yan, 2007). After incubation at 37°C with 5% CO₂ for 24 h, hepatocytes were treated with 50 ng/ml IL-6 for 24 h. This type of study usually uses a concentration between 10 and 100 ng/ml (Pascucci et al., 2000; Gubbins et al., 2003). HepG2 hepatoma line was purchased from American Type Culture Collection (Manassas, VA), and cells were maintained in Eagle's minimal essential medium containing 10% fetal bovine serum, penicillin/streptomycin, and 1 \times nonessential amino acids. HepG2 cells were usually seeded at a density of 2.5×10^5 cells/well (12-well plates) in normal medium, and treatment with IL-6 was performed 12 h after seeding. The treated cells were cultured in 1% serum-reduced medium.

Quantitative Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated with an RNA-Bee (Tel-Test Inc., Friendswood, TX) according to the manufacturer's manual, and the integrity of the RNA was confirmed by formaldehyde gel electrophoresis. Total RNA (1 μ g) was subjected to the synthesis of the first-strand cDNA in a total volume of 25 μ l with random primers and Moloney murine leukemia virus reverse transcriptase. The reactions were conducted at 25°C for 10 min, at 42°C for 50 min, and at 70°C for 10 min. The cDNAs were then diluted eight times, and quantitative PCR was conducted with TaqMan gene expression assay (Applied Biosystems, Foster City, CA). The TaqMan assay identification numbers were as follows: HCE1, Hs00275607_m1; HCE2, Hs00187279_m1; CYP3A4, Hs00604506_m1; and GAPDH, 4352934E. It should be noted that the HCE1 probe could detect both HCE1A1 and HCE1A2 transcripts. The PCR amplification was conducted in a total volume of 20 μ l containing 10 μ l of universal PCR master mixture, 1 μ l of gene-specific TaqMan assay mixture, and 6 μ l of cDNA template. Cycling profile was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, as recommended by the manufacturer. Amplification and quantification were done with the Applied Biosystems 7500 real-time PCR system.

Reporter Constructs. The HCE2 promoter reporter (–1931/+6) was kindly provided by Dr. M. E. Dolan (University of Chicago, Chicago, IL) (Wu et al., 2003). Three HCE1A2 promoter reporters were prepared to contain various lengths of HCE1A2 genomic se-

quence: HCE1A2-9224-Luc, HCE1A2-8251-Luc, and HCE1A2-7851-Luc. The HCE1A2-9224-Luc reporter was prepared first, and it contained the genomic sequence from -9133 to -40 (relative to the translation initiation codon). Two fragments (-5155 to -40 and -9224 to -5049) were initially amplified by PCR with high-fidelity Platinum *Taq*DNA polymerase and human genomic DNA as the template. The sequence (-5155 to -5049) overlapped by these two fragments contains an EcoRV site. The fragment from -5155 to -40 was generated by primers forward, 5'-tatgggacgcgttcataccac-3' and reverse, 5'-gcgaggatccgtgcagctcagagg-3' (restriction endonuclease sites are underlined). The fragment from -9224 to -5049 was generated by primers forward, 5'-taacgcgtgt-acaggatgagc-3' and reverse, 5'-tccata-ggat-ccttcagagat-3'. These two fragments were, respectively, subcloned into the pGL3 basic vector through *Mlu*I and *Bam*HI sites. To prepare the HCE1A2-9224-Luc reporter, the fragment (-9224 to -5049) was released by *Mlu*I-EcoRV digestion, and it was ligated to the construct containing the fragment from -5155 to -40, which was pretreated with *Mlu*I-EcoRV. To prepare HCE1A2-8251-Luc and HCE1A2-7851-Luc reporters, the corresponding fragments were amplified by PCR with the same reverse primer, 5'-gttcaggatatacaggtggctgttgcctatggtc-3', but with different forward primers. The forward primer for HCE1A2-8251-Luc was 5'-cacatggagctcaattccaggcctgtcactt-3', whereas the forward primer for HCE1A2-7851-Luc was 5'-agaacagagctcagtgagaaggcagcagctctgc-3'. These fragments were digested with *Sac*I and *Eco*RV and ligated to the HCE1A2-9224-Luc reporter pretreated with the same restriction endonucleases. All reporter constructs were subjected to sequence analysis.

Cotransfection Assays. Cells (HepG2) were plated in 48-well plates in DMEM supplemented with 10% fetal bovine serum at a density of 6×10^4 cells/well. Transfection was conducted by FUGENE HD (Roche Diagnostics, Indianapolis, IN). Transfection mixtures contained 100 ng of a reporter plasmid and 5 ng of thymidine kinase-*Renilla reniformis* luciferase plasmid. Cells were transfected for 12 h, and the medium was replaced with fresh medium supplemented with 1% fetal bovine serum with or without 50 ng/ml IL-6. The treatment lasted for 12 h, and then the cells were washed once with phosphate-buffered saline (PBS) and collected by scraping. The collected cells were subjected to two cycles of freeze/thaw. The reporter enzyme activities were assayed with a Dual-Luciferase reporter assay system. This system contained two substrates, which were used to determine the activities of two luciferases sequentially. The firefly luciferase activity, which represented the reporter activity, was initiated by mixing an aliquot of lysates (10 μ l) with Luciferase Assay Reagent II (Promega, Madison, WI). Then, the firefly luminescence was quenched, and the *R. reniformis* luminescence was simultaneously activated by adding Stop & Glo reagent (Promega) to the sample tubes. The firefly luminescence signal was normalized based on the *R. reniformis* luminescence signal.

Western Analysis. Cell lysates (2–20 μ g) were resolved by 7.5% SDS-polyacrylamide gel electrophoresis in a mini-gel apparatus (Bio-Rad Laboratories, Hercules, CA) and transferred electrophoretically to nitrocellulose membranes. After nonspecific binding sites were blocked with 5% nonfat milk, the blots were incubated with an antibody against HCE1, HCE2, or GAPDH. The preparation of the antibodies against HCE1 and HCE2 was described previously (Zhu et al., 2000; Xie et al., 2002). The primary antibodies were subsequently localized with goat anti-rabbit IgG conjugated with horseradish peroxidase. Horseradish peroxidase activity was detected with a chemiluminescent kit (SuperSignal West Pico; Pierce Chemical). The chemiluminescent signal was captured by Kodak Image Station 2000 (Eastman Kodak, Rochester, NY), and the relative intensities were quantified by Kodak 1D Image Analysis software.

Enzymatic Assay. Primary hepatocytes or HepG2 cells were treated with IL-6 as described above. Cells were rinsed with PBS and harvested in 300 μ l of 100 mM potassium phosphate buffer, pH 7.2. The cell suspension was sonicated by a sonifier (Branson Ultrasonics Corporation, Danbury, CT), and cell debris was removed by centrif-

ugation at 12,000g for 15 min at 4°C. The supernatant was assayed for hydrolytic activity toward *para*-nitrophenylacetate as described previously (Yang and Yan, 2007). A sample cuvette (1 ml) contained 10 to 100 μ g of microsomes in 100 mM potassium phosphate buffer, pH 7.4, and 1 mM substrate at room temperature. Reactions were initiated by adding *para*-nitrophenylacetate (10 μ l of 100 mM stock in acetonitrile), and the hydrolytic rate was recorded from an increase in absorbance at 400 nm. The extinction coefficient (E_{400}) was determined to be 13 mM⁻¹ cm⁻¹. Several controls were performed, including incubation without proteins.

Cytotoxicity Assay. HepG2 cells were seeded into 96-well plates at a density of 5000 cells/well. After an overnight incubation, the medium was replaced with fresh medium with or without 50 ng/ml IL-6 in 1% serum (half of the wells with medium alone and half of the wells with IL-6-containing medium). After an additional 12-h incubation, the cells were washed with DMEM twice and treated with clopidogrel, irinotecan, or oseltamivir at various concentrations. After the cells were treated for 24 h, the medium was replaced with fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at a final concentration of 0.5 mg/ml. After 2-h incubation at 37°C, the medium was gently decanted, and dimethyl sulfoxide (100 μ l/well) was added to dissolve formazan product. The optical density (OD) was determined at 570 nm, and the final OD values were expressed by subtracting the background reading (no seeded cells).

Other Analyses. Protein concentrations were determined with BCA protein assay (Pierce Chemical) based on albumin standard. Data are presented as mean \pm S.D. of at least three separate experiments, except where results of blots are shown, in which case a representative experiment is depicted in the figures. Statistical analysis was performed using SAS software (SAS, version 9.1; SAS Institute, Cary, NC). Significance was made according to one-way analysis of variance followed by Duncan's multiple comparison test ($p \leq 0.05$).

Results

Primary Hepatocytes Treated with IL-6 Express Markedly Lower Levels of HCE1 and HCE2 mRNA. It has been reported that hydrolytic biotransformation is decreased in patients with liver conditions such as hepatitis and cirrhosis (Thiollet et al., 1992; Gross et al., 1993; Prandota, 2005). In these conditions, the production of various proinflammatory cytokines (e.g., IL-6) is markedly increased (Frye et al., 2002; Zhang et al., 2002; Eriksson et al., 2004). To link the increased production of cytokines to the reduced hydrolysis, we tested whether IL-6 suppresses the expression of carboxylesterases HCE1 and HCE2, two major hydrolytic enzymes in the liver. Primary hepatocytes were cultured and treated with IL-6. Total RNA was isolated, and the levels of HCE1 and HCE2 were determined by qRT-PCR with *Taq*-Man probes. HCE1 has two closely related genes that encode almost identical transcripts, and the HCE1 *Taq*-Man probe recognized both transcripts. The level of CYP3A4 mRNA was monitored to serve as a positive control, because many cytokines have been shown to decrease the expression of CYP3A4 (Pascucci et al., 2000).

As shown in Fig. 1, treatment with IL-6 consistently decreased the level of HCE1, HCE2, or CYP3A4 mRNA. However, the magnitude of the decrease varied depending on genes and donors. Overall, the most decrease was detected in CYP3A4 mRNA (Fig. 1C), and the least decrease was detected in HCE1 mRNA (Fig. 1A). The level of CYP3A4 mRNA decreased by 64 to 99%, the level of HCE2 mRNA decreased by 34 to 63%, and the level of HCE1 mRNA decreased by 20

to 40% (Fig. 1). It should be noted that a regular RT-PCR with primers specific to HCE1A1 or HCE1A2 detected comparable decreases of both transcripts (data not shown). Among individual donors, the least decrease was detected with donor 2 in HCE1 and CYP3A4 mRNA, whereas donor 4 showed the least decrease in HCE2 mRNA (Fig. 1). At the basal level, CYP3A4 mRNA exhibited the largest interindividual variation, whereas HCE1 mRNA exhibited the least interindividual variation (Fig. 1). We previously tested 19 human liver microsomal samples for the abundance of HCE1 and HCE2, and we detected a larger overall interindividual variation (3- to 4-fold), although the majority of the microsomal samples exhibited a lesser variation (Tang et al., 2006).

IL-6 Reduces the Overall Hydrolytic Activity by Decreasing HCE1 and HCE2 Proteins. We next examined whether the decreases of HCE1 and HCE2 mRNA translate into decreases in the hydrolytic activity. Primary hepatocytes were treated with IL-6, and cell lysates were prepared. The overall hydrolytic activity by the lysates was determined with standard substrate *para*-nitrophenylacetate. Both HCE1 and HCE2 have been shown to hydrolyze this ester

(Xie et al., 2002). Consistent with the decreases in HCE1 and HCE2 mRNA, the hydrolysis of *para*-nitrophenylacetate was markedly decreased (Fig. 2A). To determine whether the decreased hydrolysis is due to decreased enzyme proteins, the lysates were analyzed by Western blotting for the abundance of HCE1 and HCE2. As expected, the levels of both HCE1 and HCE2 proteins were markedly decreased, and the decrease was comparable with the decrease in the hydrolytic activity (Fig. 2A, bottom). As expected, no decrease was detected in the level of GAPDH, a housekeeping gene.

To determine whether hepatoma cells respond to IL-6 sim-

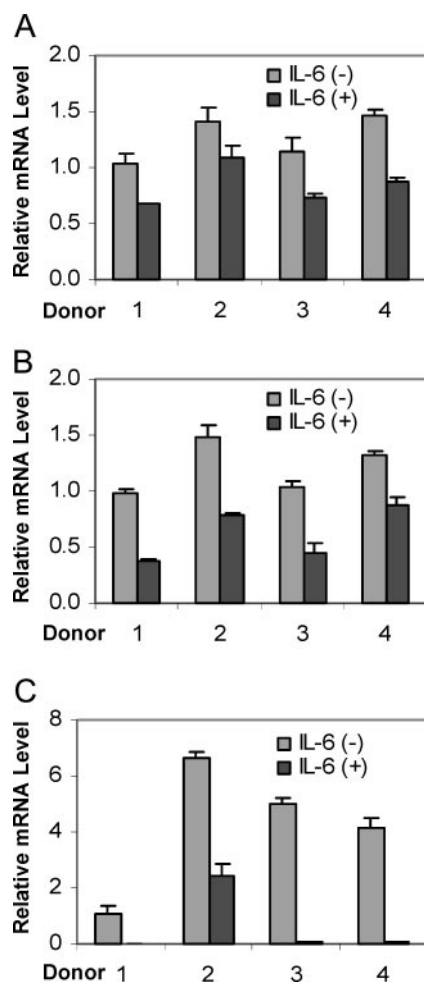


Fig. 1. Effect of IL-6 on the levels of HCE1, HCE2, and CYP3A4 mRNA in primary hepatocytes. Human primary hepatocytes were treated with 50 ng/ml IL-6 or the same volume of PBS for 24 h. Total RNAs were isolated and subjected to qRT-PCR analysis for the level of HCE1 mRNA (top), HCE2 mRNA (middle), or CYP3A4 mRNA (bottom) by TaqMan probes as described under *Materials and Methods*. The signals from each target were normalized based on the signal from GAPDH, and data are expressed as mean \pm S.D. of triplicate analyses.

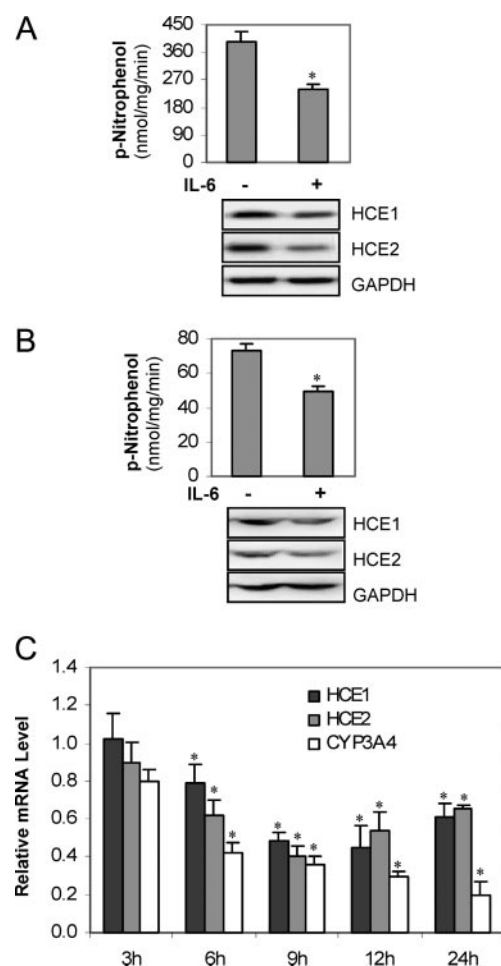


Fig. 2. Effect of IL-6 on the hydrolysis of *para*-nitrophenylacetate in primary hepatocytes and HepG2 cells, and regulated expression of HCE1, HCE2, and CYP3A4 mRNA as a function of time in HepG2 cells. A, effect of IL-6 on the hydrolysis of *para*-nitrophenylacetate in primary hepatocytes. Human hepatocytes were treated with 50 ng/ml IL-6 or with the same volume of PBS for 24 h, and cell lysates were prepared. The hydrolysis of 25 μ g/ml *para*-nitrophenylacetate was determined spectrophotometrically as described under *Materials and Methods*. To determine the abundance of HCE1 and HCE2 proteins, 2 μ g of lysates was subjected to Western analyses with an antibody against HCE1, HCE2, or GAPDH. B, effect of IL-6 on the hydrolysis of *para*-nitrophenylacetate in HepG2 cell line. HepG2 cells were treated and analyzed as described in A. However, 100 μ g of cell lysates was used for the enzymatic assay, and 20 μ g of lysates was used for the Western analyses. C, regulated expression of HCE1, HCE2, and CYP3A4 mRNA by IL-6 as a function of time. HepG2 cells were treated with IL-6 as described above; however, total RNA was prepared at various times after the initial treatment. The level of HCE1, HCE2, and CYP3A4 mRNA was determined by qRT-PCR analyses. The mRNA levels were expressed relative to those in control cells (considered as 1). All experiments described in this figure were repeated at least three times, and data are expressed as mean \pm S.D. *, $p \leq 0.05$, statistically significant decrease by IL-6 treatment.

ilarly to primary hepatocytes in terms of altered expression of carboxylesterases, HepG2 cells were treated with IL-6, and lysates were prepared and analyzed for the hydrolysis of *para*-nitrophenylacetate. Consistent with the results from primary hepatocytes, the hydrolysis of *para*-nitrophenylacetate was markedly decreased in HepG2 cells treated with IL-6 (Fig. 2B). Likewise, the protein levels of HCE1 and HCE2 were comparably decreased. It should be noted that HepG2 cells expressed a much lower HCE1 and HCE2 than primary hepatocytes, and more lysates were used for the Western analysis (2 versus 20 μ g). Likewise, the overall hydrolytic activity of HepG2 cells was much less than that of primary hepatocytes (Fig. 2, A and B).

We next performed a time course study with HepG2 cells, and qRT-PCR was used to monitor the changes of mRNA levels. The mRNA levels from IL-treated cells were normalized based on the mRNA levels from the corresponding control cells processed at various time points (expressed as 1). As shown in Fig. 2C, some decreases were detected as early as 3 h after treatment in HCE2 and CYP3A4 mRNA, although the decrease was statistically insignificant compared with that of the nontreated cells. The maximal decrease was detected at 9 h after IL-6 treatment on the levels of HCE1 and HCE2 mRNA (Fig. 2C). Thereafter, the levels of both HCE1 and HCE2 mRNA were slightly increased (remaining a statistically significant decrease), although the overall levels remained significantly lower than the levels in control cells (Fig. 2C). In contrast, a continuous decrease was detected in

CYP3A4 mRNA (Fig. 2C). It should be noted that a continuous decrease in HCE1 and HCE2 mRNA was detected when a repeated treatment with IL-6 was performed at 9 h after the initial treatment (data not shown).

IL-6 Represses the Promoter of HCE1 and HCE2. The comparable decreases between mRNA and protein levels suggest that IL-6 suppresses the expression of HCE1 and HCE2 through transcriptional repression, increased degradation of mRNA, or both. To decipher these possibilities, the effect of IL-6 on the HCE1 and HCE2 mRNA was studied in the presence of actinomycin D or DRB, two inhibitors of RNA synthesis. HepG2 cells were cotreated with IL-6 and actinomycin D or DRB for 9 h, and the levels of HCE1 and HCE2 mRNA were determined by qRT-PCR. As shown in Fig. 3A, cotreatment with an RNA synthesis inhibitor abolished the IL-6-mediated decreases on HCE1 and HCE2. It should be noted that DRB itself caused a slight decrease in HCE1 mRNA and that actinomycin D caused a moderate decrease in HCE2 mRNA. Therefore, the HCE1 data shown in Fig. 3A are from the cells treated with actinomycin D, and the HCE2 data are from the DRB treatment.

The abolition of IL-6-mediated suppression by RNA synthesis inhibitors suggests that this cytokine decreases the expression of these carboxylesterases by repressing their promoters. To test this possibility, cotransfection was performed with an HCE1 or HCE2 promoter reporter. The HCE1 reporter contains the genomic sequence corresponding to HCE1A2 promoter (HCE1A2-9224-Luc). As shown in Fig. 3C,

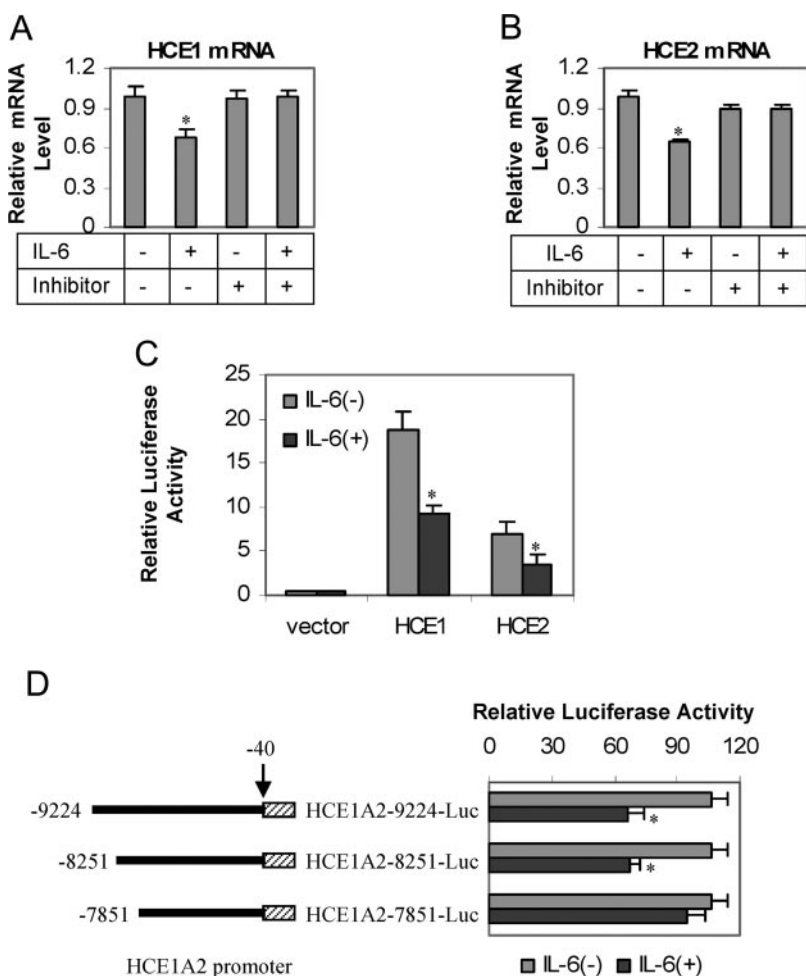


Fig. 3. Transcriptional involvement in HCE1 and HCE2 suppression by IL-6. A and B, effect of actinomycin D or DRB on the suppression of HCE1 and HCE2 mRNA. HepG2 cells were treated with 50 ng/ml IL-6 for 9 h in the absence or presence of 0.5 μ M actinomycin D or 5 μ M DRB. Total RNA was prepared and analyzed for the level of HCE1 and HCE2 mRNA by qRT-PCR. C, repression of HCE1 and HCE2 promoter reporters. HepG2 cells were transiently transfected by FuGENE HD with a mixture containing 100 ng of HCE1A2-9224-Luc or HCE2-Luc, along with 5 ng of the TK-R. *reniformis* luciferase plasmid. After incubation at 37°C for 12 h, the transfected cells were treated with 50 ng/ml IL-6 or the same volume of PBS for 12 h. Luciferase activities were determined with a Dual-Luciferase reporter assay system, and the reporter activity was normalized based on the *R. reniformis* luminescence signal. D, differential repression of HCE1A2-9224-Luc, HCE1A2-8251-Luc, and HCE1A2-7851-Luc. HepG2 cells were transfected and treated as in C. In addition to HCE1A2-9224-Luc, HCE1A2-8251-Luc, and HCE1A2-7851-Luc reporters were used. The activities are expressed as percentages of that of HCE1A2-9224-Luc. Three independent experiments were performed. *, $p \leq 0.05$, statistically significant decrease by IL-6 treatment.

treatment with IL-6 resulted in decreased activities of both HCE1 and HCE2 reporters by ~40%. To locate the sequence in the HCE1 promoter that supports the repression, two deletion mutants of the HCE1-reporter (5' end) were prepared and tested for the responsiveness to IL-6. As shown in Fig. 3D, the repression was detected with the HCE1-8251-Luc reporter but not HCE1-7851-Luc, suggesting that the sequence responsible for the repression is located at the region between -8251 and -7851.

Alteration of Cellular Responsiveness to Clopidogrel, Irinotecan, and Oseltamivir. We next examined the pharmacological/toxicological consequences of the reduced expression of carboxylesterases by IL-6. It has been reported that HCE1 and HCE2 differ markedly in the hydrolysis of antiplatelet agent clopidogrel, anticancer agent irinotecan, and anti-influenza agent oseltamivir (Wu et al., 2002; Shi et al., 2006; Tang et al., 2006). HCE1 rapidly hydrolyzes clopidogrel and oseltamivir, whereas HCE2 rapidly hydrolyzes irinotecan. More importantly, hydrolysis of clopidogrel represents detoxication, whereas hydrolysis of irinotecan and oseltamivir increases cytotoxicity. It was assumed that IL-6 treatment would increase cytotoxicity of clopidogrel, and the opposite would be true with irinotecan and oseltamivir. HepG2 cells were first treated with IL-6 for 12 h, washed twice, and then treated with a drug at various concentrations. After an additional 24-h incubation, cell viability was determined by MTT assay.

As shown in Fig. 4, IL-6 pretreatment alone caused no difference in the cell viability (no drug treatment); however, pretreatment followed by a drug treatment caused statistically significant changes with all drugs and all concentrations compared with nonpretreated cells (Fig. 4). When exposed to clopidogrel as low as 3 μ M, for example, the cells pretreated with IL-6 exhibited a 40% decrease in cell viability (Fig. 4A). The decreased viability by IL-6 pretreatment was consistent with the observation that hydrolysis of clopidogrel represents detoxication (Tang et al., 2006). In contrast, IL-6 pretreatment protected against oseltamivir-induced cytotoxicity. For example, when oseltamivir was assayed at 3 μ M, the IL-6-pretreated cells had a relative viability of 150% of the nontreated cells (Fig. 4B). Interestingly, oseltamivir at low concentrations (3 and 30 μ M) caused a statistically significant increase in cell viability among IL-6-pretreated cells (Fig. 2B). Nevertheless, the increased viability in IL-6-pretreated cells (decreased oseltamivir hydrolysis) is consistent with the previous observation that the hydrolytic metabolite but not the parent drug of oseltamivir causes cell toxicity (Shi et al., 2006). Finally, irinotecan is an anticancer prodrug, and only the hydrolytic metabolite has anticancer activity (Wu et al., 2002). Irinotecan decreased cell viability by 45% or higher compared with nontreated cells (without IL-6 pretreatment), but IL-6 pretreatment completely blocked this effect on cell viability at 0.3 μ M, and it attenuated the reduction in viability with higher irinotecan exposures (Fig. 4C). For clarity, some of the statistical significances, particularly those showing obvious differences, are not labeled in this figure. An example is the statistically significant difference between irinotecan-treated and nontreated cells that were not pretreated with IL-6 (Fig. 4C).

We next examined morphological changes in cells pretreated with IL-6 in response to a drug. Under bright field, cells pretreated with IL-6 were spread, and the projects were

well extended when exposed to oseltamivir (Fig. 5, top right). In contrast, cells without IL-6 pretreatment were rounded, isolated, and swollen with the presence of vesicles in the cytoplasm (Fig. 5, top left). Conversely, when exposed to clopidogrel, cells pretreated with IL-6 exhibited profound morphological changes (e.g., aggregation), whereas cells without IL-6 pretreatment showed normal appearance (Fig. 5, middle). As for irinotecan, cells without IL-6 pretreatment were isolated and shrank, whereas cells pretreated with IL-6 were morphologically normal (Fig. 5, bottom). It should be emphasized that the observed changes were opposite to the changes of cells transfected by HCE1 (Shi et al., 2006; Tang et al., 2006) or HCE2 (Wu et al., 2002). The morphological analyses were performed 48 h after the treatment (drug medium was replaced with fresh drug-containing medium at 24 h), whereas the cell viability was determined 24 h after the initial treatment with a drug (Fig. 4).

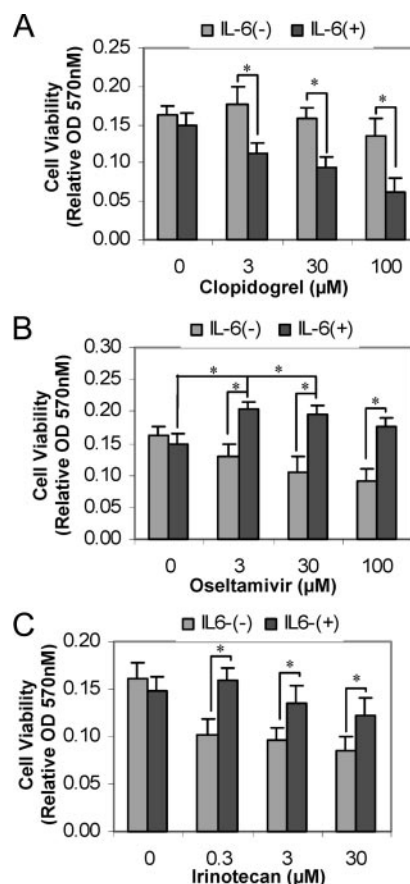


Fig. 4. Effect of IL-6 on the cellular responsiveness to clopidogrel (A), irinotecan (B), and oseltamivir (C). HepG2 cells were seeded into 96-well plates at a density of 5000 cells/well. After an overnight incubation, 50 ng/ml IL-6 in 1% serum medium was added to half of the wells, and the treatment lasted for 12 h. Thereafter, the cells were washed with DMEM, and then they were treated with clopidogrel, irinotecan, or oseltamivir at various concentrations. After an additional 24-h incubation, MTT was added to each well at a final concentration of 0.5 mg/ml. After 2-h incubation at 37°C, the medium was gently decanted, and dimethyl sulfoxide (150 μ l/well) was added to dissolve formazan product. The OD was determined at 570 nm, and the final OD values were expressed by subtracting the background reading (no seeded cells). The data are from three independent experiments, and they are expressed as mean \pm S.D. *, $p \leq 0.05$, statistically significant difference between columns specified by lines.

Discussion

Carboxylesterases constitute a class of hydrolytic enzymes that play important roles in the metabolism of drugs and other xenobiotics. In patients with liver conditions such as cirrhosis, the secretion of proinflammatory cytokines (e.g., IL-6) is increased, and the hydrolysis of many drugs is decreased (Thiollet et al., 1992; Gross et al., 1993; Frye et al., 2002; Zhang et al., 2002). In this study, we have provided a molecular explanation linking cytokine secretion directly to the decreased capacity of hydrolytic biotransformation. In both primary hepatocytes and HepG2 cells, treatment with IL-6 causes significant decreases in the expression of HCE1 and HCE2, two major carboxylesterases in the liver. The decreased expression occurs at both mRNA and protein levels, and it is confirmed by enzymatic assay. In cells treated with IL-6, the transcriptional activity of both HCE1 and HCE2 promoters is markedly repressed.

In addition to decreased expression of carboxylesterases, IL-6 pretreatment profoundly alters the cellular responsiveness to various ester drugs, including clopidogrel, irinotecan, and oseltamivir. The alteration is clearly achieved by decreasing the expression of HCE1 or HCE2. Multiple washes are performed to remove any residual IL-6 before treatment with a drug. More importantly, pretreatment with IL-6 alters the cellular responsiveness in an opposite manner of overex-

pression of HCE1 and HCE2. For example, transfection of HCE1 decreases clopidogrel-induced cytotoxicity (Tang et al., 2006), whereas pretreatment with IL-6 increases cytotoxicity (Fig. 4A). Conversely, transfection of HCE1 increases the cytotoxicity of oseltamivir (Shi et al., 2006), and the cytotoxicity is abolished by IL-6 pretreatment (Fig. 4B). Likewise, hydrolysis of irinotecan by HCE2 leads to increased cytotoxicity (Wu et al., 2002), and IL-6 pretreatment protects against cytotoxicity (Fig. 4C). It should be noted that all ester drugs are tested at submicromolar or micromolar concentrations, and the majority of these concentrations are pharmacologically relevant.

IL-6 suppresses the expression of both HCE1 and CYP3A4, and it probably presents a complex scenario on the pharmacological behaviors of clopidogrel. This antithrombogenic agent undergoes hydrolytic and oxidative metabolism. The hydrolysis is catalyzed by HCE1 (Tang et al., 2006), whereas the oxidation is catalyzed by CYP3A4 and CYP3A5 (Savi et al., 2000). More importantly, the oxidized but not hydrolytic metabolite exerts antithrombogenic activity, and hydrolysis of the ester bond represents inactivation (Savi et al., 2000). It is assumed that decreased expression of HCE1 would increase the amount of the parent drug for oxidation and favor therapeutic outcome. Conversely, decreased expression of CYP3A4 would decrease the oxidative metabolism and reduce its therapeutic activation. The ultimate effect, however, probably depends on the relative magnitude of decreased expression between HCE1 and CYP3A4 and the relative rate between these two types of metabolism. In this study, we have shown that the expression of CYP3A4 is decreased more than HCE1 in IL-6-treated cells (Fig. 1, A and C), suggesting that the oxidation is affected to a greater extent. Alternatively, hydrolysis usually proceeds much faster than oxidation catalyzed by P450s (Parkinson, 2001); thus, decreased hydrolysis during IL-6 exposure would weigh more over decreased oxidation, and prolong the therapeutic effect of clopidogrel. In support of the second possibility, patients with liver cirrhosis (i.e., IL-6 exposure) show increased bleeding time accompanied by decreased formation of the hydrolytic metabolite (Slugg et al., 2000).

In contrast to clopidogrel, hydrolysis of oseltamivir is required for its antiviral activity (Oxford et al., 2003). The hydrolytic metabolite is a potent inhibitor of the neuraminidase of influenza virus, and this sialidase plays important roles in the process of viral entry and release (Wagner et al., 2002; Ohuchi et al., 2006). It is expected that decreased hepatic hydrolysis by IL-6 probably results in decreased therapeutic activity of oseltamivir. In patients with liver cirrhosis, both C_{\max} and area under the curve_{0-∞} values are decreased almost by 20% (Snell et al., 2005), but the precise therapeutic significance of such a decrease remains to be determined. In contrast, decreased hepatic hydrolysis of oseltamivir may have toxicological significance. It has been reported recently that some flu patients taking oseltamivir show neurobehavioral changes (Food and Drug Administration, 2007). Although the precise involvement of oseltamivir in the neurotoxicity remains to be established, it is tempting to speculate that patients who develop neurological symptoms may have increased levels of oseltamivir in the brain, and decreased hepatic hydrolysis probably contributes to such an increase. In humans, there are several mechanisms that may support reduced hepatic hydrolysis of oseltamivir.

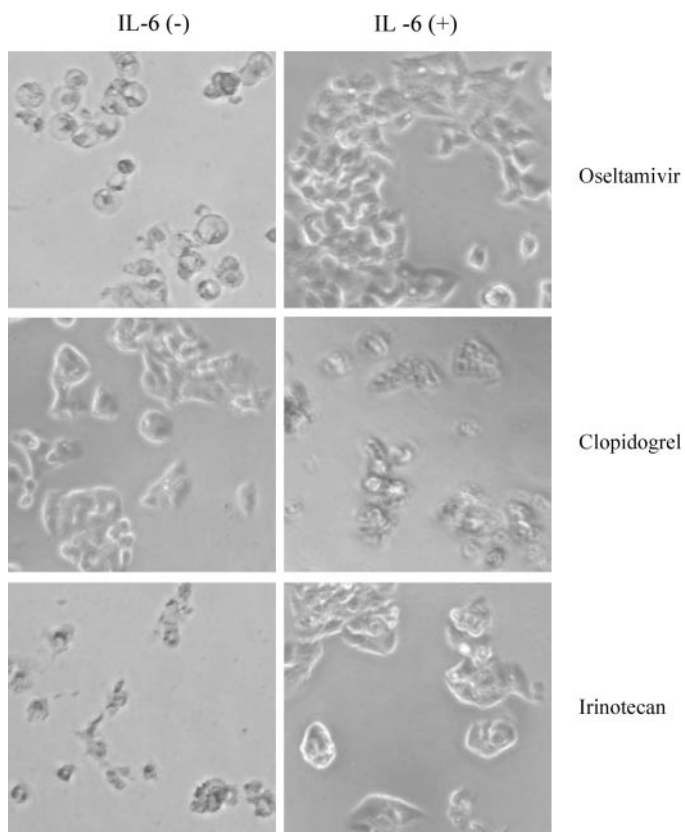


Fig. 5. Morphological analyses HepG2 cells were seeded into 96-well plates at a density of 5000 cells/well. After an overnight incubation, 50 ng/ml IL-6 in 1% serum medium was added to half of the wells, and the treatment lasted for 12 h. Thereafter, the cells were washed with DMEM twice, and then cells were treated with 100 μ M oseltamivir, 100 μ M clopidogrel, or 3 μ M irinotecan in full medium. The drug medium was replaced with fresh drug-containing medium at 24 h. After an additional 24-h incubation, images were taken under bright field (250 \times).

First, certain drugs (e.g., clopidogrel) concurrently administered may profoundly inhibit oseltamivir hydrolysis (Shi et al., 2006). Second, people express polymorphic variants of HCE1 with marked decreases in the hydrolysis of oseltamivir (Shi et al., 2006). And finally, excessive production of cytokines (e.g., IL-6) down-regulates the expression of HCE1. In support of the last possibility, influenza viral infection has been shown to increase the secretion of various proinflammatory cytokines, including IL-6 (Aiba et al., 2001).

Like oseltamivir, irinotecan is an ester prodrug, and the hydrolytic metabolite exerts potent anticancer activity (Masuda et al., 1996). In contrast to oseltamivir, irinotecan is hydrolyzed by HCE2 but not HCE1 (Wu et al., 2002). Although the liver expresses high levels of HCE2, it has been suggested that hydrolytic metabolite generated locally contributes significantly to its antitumor activity (Rowinsky et al., 1994; Kojima et al., 1998). In support of this notion, gallbladder carcinoma expresses little HCE2, and it does not respond to irinotecan (Alberts et al., 2002). Interestingly, several investigators have shown that the expression of HCE2 is decreased in tumors (Guichard et al., 1999; Xie et al., 2002). However, the precise mechanism on the tumor-associated decrease remains to be determined. In contrast, the expression of various proinflammatory cytokines, including IL-6, is elevated in tumor tissues (Nakano et al., 1999; Charles et al., 2006). In this study, we have demonstrated in hepatocytes and hepatoma cells treated with IL-6 a marked decrease the expression of HCE2 (Figs. 1B and 2C). Whether increased expression of IL-6 is responsible for the tumor-associated decrease in HCE2 expression remains to be determined. CYP3A4 reportedly metabolizes irinotecan, and the oxidized metabolites can be converted to the active metabolite by HCE2. However, the conversion is much lesser effective than that of irinotecan (Sanghani et al., 2004). Therefore, the greater suppression of CYP3A4 than HCE2 expression (Fig. 1) probably increases the availability of parent drug for hydrolysis.

Proinflammatory cytokines such as IL-6 have been shown to suppress the expression of many drug-metabolizing enzymes, particularly P450 enzymes (Pascucci et al., 2000; Frye et al., 2002; Sunman et al., 2004). In this study, we have shown that IL-6 causes marked decreases in the expression of HCE1 and HCE2, further increasing the spectrum of repressed enzymes. Although IL-6 exerts a broad suppressive activity, the mechanisms supporting the suppression may vary from target to target. In fact, we have observed several major differences on the suppressed expression of carboxylesterases and CYP3A4. First, the suppression of CYP3A4 is much more profound than that of either HCE1 or HCE2 (Fig. 1). Second, the magnitude of suppression of carboxylesterases is similar between primary hepatocytes and HepG2 cells, whereas the suppression of CYP3A4 is much higher in primary hepatocytes than that in HepG2 cells (Figs. 1C and 2C). Third, a continuous decrease in CYP3A4 mRNA occurs during the full time course of the study (up to 24 h). In contrast, the maximum decrease in HCE1 and HCE2 mRNA occurs 9 h after the treatment (Fig. 2C). It should be noted that repeated treatment with IL-6 leads to more persistent decreases in HCE1 and HCE2 mRNA (data not shown).

It seems that suppressed expression of HCE1 and HCE2 is achieved primarily through transcriptional repression. We have presented several lines of evidence that directly support

this possibility. First, the IL-6-mediated suppression is abolished by RNA synthesis inhibitors (Fig. 3A), excluding an involvement of post-transcriptional mechanism. Second, the promoters of HCE1 and HCE2 are markedly repressed in cells treated with IL-6 (Fig. 3B). More importantly, the repression of the promoters is comparable with the extent of decreased mRNA and proteins (Figs. 1–3), providing direct evidence that transcriptional repression is responsible for the suppressed expression of the carboxylesterases. And finally, we have located a genomic sequence in the HCE1A2 gene that supports the transcriptional repression. HCE1A2-Luc(–8213/–40) but not HCE1A2-Luc(–7813/–40) responded to IL-6 (Fig. 3C), suggesting that the sequence (–8213 to –7813) contains a response element(s) that supports the response to IL-6.

In summary, our work points to several important conclusions. First, we have shown that IL-6 causes marked decreases in the expression of HCE1 and HCE2, suggesting that proinflammatory cytokines exert a broad suppression on the expression of various types of drug-metabolizing enzymes. Second, we have observed several differences in the suppressed expression between carboxylesterases and CYP3A4, suggesting that multiple mechanisms support the action of IL-6 and that these mechanisms operate independently or cooperatively, depending on a target gene. And finally, pretreatment with IL-6 profoundly alters the cellular responsiveness to ester drugs such as oseltamivir, suggesting that suppressed expression of carboxylesterases has profound pharmacological and toxicological consequences, particularly with those drugs that are hydrolyzed in an isoform-specific manner.

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References

- Aiba H, Mochizuki M, Kimura M, and Hojo H (2001) Predictive value of serum interleukin-6 level in influenza virus-associated encephalopathy. *Neurology* **57**: 295–299.
- Alberts SR, Fishkin PA, Burgart LJ, Cera PJ, Mahoney MR, Morton RF, Johnson PA, Nair S, and Goldberg RM (2002) North Central Cancer Treatment Group. CPT-11 for bile-duct and gallbladder carcinoma: a phase II North Central Cancer Treatment Group (NCCTG) study. *Int J Gastrointest Cancer* **32**:107–114.
- Blokzijl H, Vander Borghst S, Bok LI, Libbrecht L, Geuken M, van den Heuvel FA, Dijkstra G, Roskams TA, Moshage H, Jansen PL, et al. (2007) Decreased P-glycoprotein (P-gp/MDR1) expression in inflamed human intestinal epithelium is independent of PXR protein levels. *Inflamm Bowel Dis* **13**:710–720.
- Charles KA, Rivory LP, Brown SL, Liddle C, Clarke SJ, and Robertson GR (2006) Transcriptional repression of hepatic cytochrome P450 3A4 gene in the presence of cancer. *Clin Cancer Res* **12**:7492–7497.
- Eriksson AS, Gretzer C, and Wallerstedt S (2004) Elevation of cytokines in peritoneal fluid and blood in patients with liver cirrhosis. *Hepatogastroenterology* **51**: 505–509.
- Food and Drug Administration (2007) *FDA Patient Safety News: A Video News Show for Health Professionals. Caution on Neuropsychiatric Events with Tamiflu*. United States Food and Drug Administration, Washington DC. Available at: <http://www.accessdata.fda.gov/psn/transcript.cfm?show=59#3>.
- Frye RF, Schneider VM, Frye CS, and Feldman AM (2002) Plasma levels of TNF- α and IL-6 are inversely related to cytochrome P450-dependent drug metabolism in patients with congestive heart failure. *J Card Fail* **8**:315–319.
- Gross V, Treher E, Haag K, Neis W, Wiegand U, and Scholmerich J (1993) Angiotensin-converting enzyme (ACE)-inhibition in cirrhosis. Pharmacokinetics and dynamics of the ACE-inhibitor cilazapril (Ro 31-2848). *J Hepatol* **17**:40–47.
- Gubbins PO, Melchert RB, McConnell SA, Franks AM, Penzak SR, and Gurley BJ (2003) Effect of interleukin 6 on the hepatic metabolism of itraconazole and its metabolite hydroxyitraconazole using primary human hepatocytes. *Pharmacology* **67**:195–201.
- Guichard S, Terret C, Hennebelle I, Lochon I, Chevreau P, Fretigny E, Selves J, Chatelut E, Bugat R, and Canal P (1999) CPT-11 converting carboxylesterase and topoisomerase activities in tumor and normal colon and liver tissues. *Br J Cancer* **80**:364–370.

- Kojima A, Hackett NR, Ohwada A, and Crystal RG (1998) In vivo human carboxylesterase cDNA gene transfer to activate the prodrug CPT-11 for local treatment of solid tumors. *J Clin Invest* **101**:1789–1796.
- Masuda N, Kudoh S, and Fukuoaka M (1996) Irinotecan (CPT-11): pharmacology and clinical applications. *Crit Rev Oncol Hematol* **24**:3–26.
- Nakano Y, Kobayashi W, Sugai S, Kimura H, and Yagihashi S (1999) Expression of tumor necrosis factor- α and interleukin-6 in oral squamous cell carcinoma. *Jpn J Cancer Res* **90**:858–866.
- Ohuchi M, Asaoka N, Sakai T, and Ohuchi R (2006) Roles of neuraminidase in the initial stage of influenza virus infection. *Microbes Infect* **8**:1287–1293.
- Oxford JS, Mann A, and Lambkin R (2003) A designer drug against influenza: the NA inhibitor oseltamivir (Tamiflu). *Expert Rev Anti Infect Ther* **1**:337–342.
- Parkinson A (2001) Biotransformation of xenobiotics, in *The Casarett and Doull's Toxicology: The Basic Science of Poisons* (Klaassen CD ed) pp 139–162, McGraw-Hill Companies, New York.
- Pascussi JM, Gerbal-Chaloin S, Pichard-Garcia L, Daujat M, Fabre JM, Maurel P, and Vilarem MJ (2000) Interleukin-6 negatively regulates the expression of pregnane X receptor and constitutively activated receptor in primary human hepatocytes. *Biochem Biophys Res Commun* **274**:707–713.
- Poso A and Honkakoski P (2006) Ligand recognition by drug-activated nuclear receptors PXR and CAR: structural, site-directed mutagenesis and molecular modeling studies. *Mini Rev Med Chem* **6**:937–947.
- Prandota J (2005) Important role of proinflammatory cytokines/other endogenous substances in drug-induced hepatotoxicity: depression of drug metabolism during infections/inflammation states, and genetic polymorphisms of drug-metabolizing enzymes/cytokines may markedly contribute to this pathology. *Am J Ther* **12**:254–261.
- Remick DG, Newcomb DE, Bolgos GL, and Call DR (2000) Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide versus cecal ligation and puncture. *Shock* **13**:110–116.
- Rowinsky EK, Grochow LB, Ettinger DS, Sartorius SE, Lubejko BG, Chen TL, Rock MK, and Donehower RC (1994) Phase I and pharmacological study of the novel topoisomerase I inhibitor 7-ethyl-10-[4-(1-piperidino)-1 piperidino]carbonyloxycamptothecin (CPT-11) administered as a ninety-minute infusion every 3 weeks. *Cancer Res* **54**:427–436.
- Sanghani SP, Quinney SK, Fredenburg TB, Davis WI, Murry DJ, and Bosron WF (2004) Hydrolysis of irinotecan and its oxidative metabolites, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin and 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxycamptothecin, by human carboxylesterases CES1A1, CES2, and a newly expressed carboxylesterase isoenzyme, CES3. *Drug Metab Dispos* **32**:505–511.
- Satoh T and Hosokawa M (2006) Structure, function and regulation of carboxylesterases. *Chem Biol Interact* **162**:195–211.
- Savi P, Pereillo JM, Uzabiaga MF, Combalbert J, Picard C, Maffrand JP, Pascal M, and Herbert JM (2000) Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* **84**:891–896.
- Schwer H, Langmann T, Daig R, Becker A, Aslanidis C, and Schmitz G (1997) Molecular cloning and characterization of a novel putative carboxylesterase, present in human intestine and liver. *Biochem Biophys Res Commun* **233**:117–120.
- Shi D, Yang J, Yang D, LeCluyse EL, Black C, You L, Akhlaghi F, and Yan B (2006) Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and the activation is inhibited by antiplatelet agent clopidogrel. *J Pharmacol Exp Ther* **319**:1477–1484.
- Slugg PH, Much DR, Smith WB, Vargas R, Nichola P, and Necciari J (2000) Cirrhosis does not affect the pharmacokinetics and pharmacodynamics of clopidogrel. *J Clin Pharmacol* **40**:396–401.
- Snell P, Dave N, Wilson K, Rowell L, Weil A, Galitz L, and Robson R (2005) Lack of effect of moderate hepatic impairment on the pharmacokinetics of oral oseltamivir and its metabolite oseltamivir carboxylate. *Br J Clin Pharmacol* **59**:598–601.
- Tang M, Mukundan M, Yang J, Charpentier N, LeCluyse EL, Black C, Yang D, Shi D, and Yan B (2006) Antiplatelet agents aspirin and clopidogrel are hydrolyzed by distinct carboxylesterases, and clopidogrel is transesterified in the presence of ethyl alcohol. *J Pharmacol Exp Ther* **319**:1467–1476.
- Tanimoto K, Kaneyasu M, Shimokuni T, Hiyama K, and Nishiyama M (2007) Human carboxylesterase 1A2 expressed from carboxylesterase 1A1 and 1A2 genes is a potent predictor of CPT-11 cytotoxicity in vitro. *Pharmacogenet Genomics* **17**:1–10.
- Thiollet M, Funck-Brentano C, Grange JD, Midavaine M, Resplandy G, and Jaillon P (1992) The pharmacokinetics of perindopril in patients with liver cirrhosis. *Br J Clin Pharmacol* **33**:326–328.
- Villa P, Sartor G, Angelini M, Sironi M, Conni M, Gnocchi P, Isetta AM, Grau G, Buurman W, van Tits L, et al. (1995) Pattern of cytokines and pharmacomodulation in sepsis induced by cecal ligation and puncture compared with that induced by endotoxin. *Clin Diagn Lab Immunol* **2**:549–553.
- Wagner R, Matrosovich M, and Klenk HD (2002) Functional balance between hemagglutinin and neuraminidase in influenza virus infections. *Rev Med Virol* **12**:159–166.
- Wait R, Chiesa G, Parolini C, Miller I, Begum S, Brambilla D, Galluccio L, Ballerio R, Eberini I, and Gianazza E (2005) Reference maps of mouse serum acute-phase proteins: changes with LPS-induced inflammation and apolipoprotein A-I and A-II transgenes. *Proteomics* **5**:4245–4253.
- Wu MH, Chen P, Remo BF, Cook EH Jr, Das S, and Dolan ME (2003) Characterization of multiple promoters in the human carboxylesterase 2 gene. *Pharmacogenetics* **13**:425–435.
- Wu MH, Yan B, Humerickhouse R, and Dolan ME (2002) Irinotecan activation by human carboxylesterases in colorectal adenocarcinoma cells. *Clin Cancer Res* **8**:2696–2700.
- Xie M, Yang D, Liu L, Xue B, and Yan B (2002) Human and rodent carboxylesterases: immunorelatedness, overlapping substrate specificity, differential sensitivity to serine enzyme inhibitors, and tumor-related expression. *Drug Metab Dispos* **30**:541–547.
- Yang J and Yan B (2007) Photochemotherapeutic agent 8-methoxypsoralen induces cytochrome P450 3A4 and carboxylesterase HCE2: evidence on an involvement of the pregnane X receptor. *Toxicol Sci* **95**:13–22.
- Zhang W, Yue B, Wang GQ, and Lu SL (2002) Serum and ascites levels of macrophage migration inhibitory factor, TNF- α and IL-6 in patients with chronic virus hepatitis B and hepatitis cirrhosis. *Hepatobiliary Pancreat Dis Int* **1**:577–580.
- Zhu W, Song L, Zhang H, Matoney L, LeCluyse E, and Yan B (2000) Dexamethasone differentially regulates expression of carboxylesterase genes in humans and rats. *Drug Metab Dispos* **28**:186–191.

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