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Down-regulation of cytochrome P450 2C8 by 3-methylcholanthrene in human hepatocellular carcinoma cell lines

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Abstract: The marked induction of cytochromes P450 such as CYP1A1 caused by polycyclic aromatic hydrocarbons (PAHs) like 3-methylcholanthrene (MC) is often accompanied by suppression of other hepatic P450s. The molecular mechanisms, functional consequences, and human relevance of P450 down-regulation by PAHs are poorly understood. MC suppresses mRNA levels for CYP2C8, an important human P450, in cultured human hepatocytes. To avoid hepatocyte lot-to-lot variability, we assessed *CYP2C8* regulation by MC in HepaRG cells, a terminally differentiated human hepatocellular carcinoma cell line that maintains high P450 expression. MC strongly induced CYP1A1 mRNA levels and markedly down-regulated CYP2C8 mRNA levels in HepaRG cells. Although MC also suppressed CYP2C8 mRNA levels in the HepG2 human hepatocellular carcinoma cell line, basal *CYP2C8* expression was extremely low. HepaRG cells appear to be an appropriate model system for studying the mechanisms and functional consequences of *CYP2C8* down-regulation by PAHs.

Key words: cytochrome P450; CYP2C8; drug metabolism; polycyclic aromatic hydrocarbons; 3-methylcholanthrene; HepG2 cells; HepaRG cells; aryl hydrocarbon receptor

Introduction

The aryl hydrocarbon receptor (AHR)-mediated induction of drug-metabolizing enzymes such as cytochrome P450 1A1 (CYP1A1) is a well-characterized response to polycyclic aromatic hydrocarbon (PAH) exposure. Much less well understood, in terms of molecular mechanisms and functional consequences, is the down-regulation of certain constitutive hepatic P450s that can accompany the pronounced CYP1A1 induction by PAHs (Riddick et al. 2003). Treatment of male rats with 3-methylcholanthrene (MC), a model PAH and carcinogen, suppresses hepatic expression and activity of CYP2C11 (Jones and Riddick 1996), a male-specific enzyme regulated at least partly by a signal transducer and activator of transcription-5b (STAT5b) pathway triggered by pulsatile pituitary growth hormone (GH) secretion (Park and Waxman 2001). The *CYP2C11* suppression response seems to occur at the transcriptional level (Bhathena et al. 2002; Lee and Riddick 2000) with a requirement for AHR involvement (Bhathena et al. 2002; Safa et al. 1997). We also showed that MC interferes with the ability of GH to stimulate hepatic *CYP2C11* expression in the liver of hypophysectomized male rats (Timsit and Riddick 2000), but there was no apparent effect of MC on GH-stimulated STAT5b signaling in rat liver or H4IIE rat hepatoma cells (Timsit and Riddick 2002).

A close relative to rat CYP2C11 is human CYP2C8, a genetically polymorphic P450 that accounts for approximately 7% of the total P450 content of human liver and plays a key role in the metabolism of arachidonic acid and all-*trans*-retinoic acid as well as several drugs such as amodiaquine, repaglinide, rosiglitazone, pioglitazone, and paclitaxel (Lai et al. 2009). The basal hepatic expression of *CYP2C8* is primarily driven by hepatocyte nuclear factor-4 α (HNF-4 α) and it is inducible by activators of the constitutive androstane receptor (CAR), pregnane X receptor (PXR), and glucocorticoid receptor (Chen and Goldstein 2009; Ferguson et al. 2005). Although AHR agonists were reported to not alter *CYP2C8* expression in the HepG2 human

hepatocellular carcinoma cell line (Westerink and Schoonen 2007), MC was found to down-regulate CYP2C8 mRNA levels in primary human hepatocyte cultures (Ning et al. 2008; Richert et al. 2009).

In an attempt to identify a cell culture system for mechanistic studies of *CYP2C8* down-regulation by PAHs that does not suffer from the lot-to-lot variability characteristic of hepatocytes, the goal of the present study was to compare the expression of *CYP2C8* and its regulation by MC in two human hepatocellular carcinoma cells lines: HepG2 and HepaRG. As an easily cultured continuous line, HepG2 ranks among the most commonly used human liver-derived cell lines; however, an important limitation of these cells in drug metabolism studies is their very low expression of many P450s. HepaRG, a terminally differentiated non-continuous human hepatocellular carcinoma cell line, represents a potential surrogate to primary human hepatocytes for drug metabolism studies. Under specific culture conditions, HepaRG cells maintain relatively high basal expression of multiple P450s and show robust induction in response to activators of AHR, CAR, and PXR (Antherieu et al. 2010; Hart et al. 2010).

Materials and Methods

Cell culture

HepG2 cells (HB-8065) were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown as monolayer cultures in α -minimum essential medium supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA) and maintained in an atmosphere of 5% CO₂ and 95% air at 37°C. Cells were plated in 55-cm² dishes and grown to 60% confluence, at which time cells were exposed to MC (1 or 5 μ M) or dimethylsulfoxide (DMSO) vehicle (0.5%). Although we used a low DMSO concentration, the known ability of DMSO to impact P450 expression represents a minor limitation (Nishimura et

al. 2003). After treatment periods of 24 and 48 h, cells were harvested and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method using Tri-Reagent (Sigma-Aldrich, St. Louis, MO).

Differentiated cryopreserved HepaRG cells (HPR116) were obtained from Biopredic International (Overland Park, KS). Upon thawing, cells were initially suspended in Williams E medium with GlutaMAX (Invitrogen) and the thawing/plating/general purpose supplement (Biopredic) and seeded at a density of 480,000 cells per well in 24-well collagen I-coated plates (BD Biosciences, Bedford, MA). The medium was replaced with Williams E medium with GlutaMAX (Invitrogen) and the maintenance/metabolism supplement (Biopredic) at one, four, and six days after seeding. On the seventh day, cells were exposed to MC (1 or 5 μ M) or 0.5% DMSO vehicle. After treatment periods of 24 and 48 h, cells were harvested and total RNA was isolated using RNeasy Mini Kits (Qiagen Inc., Valencia, CA).

Analysis of mRNA levels by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Levels of mRNA encoding CYP1A1 and CYP2C8, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference standard, were determined by the comparative threshold cycle relative quantitation method on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The following Taqman Gene Expression Assay kits (Applied Biosystems) were used for amplification and detection: CYP1A1 (Hs00153120_m1), CYP2C8 (Hs00946140_g1), and GAPDH (4333764F). Cycling conditions were an initial cycle of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All samples were analyzed in triplicate within an assay and each assay was repeated to ensure consistency.

For comparative purposes, the above RT-PCR analysis was also run with three human liver RNA samples (K15, K16, K28) randomly selected from a tissue bank maintained at the University of Toronto. The livers were originally obtained at the time of cadaveric kidney donation, and information on the donors has been reported (Sumida et al. 1999).

Statistical analysis

All values shown are the mean $\pm$ SD. Statistical tests were conducted on the original data rather than the derived data shown as percent of control. Data were analyzed by randomized-design one-way ANOVA followed by Newman-Keuls multiple comparisons test. In cases where heterogeneity of variance was encountered, data were analyzed by the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. A result was considered statistically significant if $P < 0.05$.

Results and Discussion

For mechanistic and functional investigations of *CYP2C8* down-regulation by PAHs, it is desirable for a cell culture model to display high basal expression of *CYP2C8*, preferably with levels approaching that seen in human liver. We compared *CYP2C8* mRNA levels in HepaRG and HepG2 cells to the levels seen in three randomly selected human liver samples (Fig. 1). The human liver samples showed a nearly 11-fold range in *CYP2C8* mRNA levels. *CYP2C8* mRNA levels in HepaRG and HepG2 cells were approximately 16% and less than 0.01%, respectively, of the mean level for the human liver samples. *CYP2C8* mRNA levels in HepaRG cells are closely aligned with that seen for sample K28, the human liver showing the lowest *CYP2C8* expression from among the three selected samples. This finding is consistent with the overall greater transcriptomic similarity of HepaRG cells, as opposed to HepG2 cells, to primary human

hepatocytes and human liver (Hart et al. 2010).

We chose to expose cells to MC concentrations of 1 and 5 μ M for time periods of 24 and 48 h. These treatment conditions are known to activate the AHR pathway and trigger pronounced CYP1A1 induction in both HepG2 (Roberts et al. 1990) and HepaRG (Attignon et al. 2017) cells. We also used trypan blue exclusion assays to characterize the sensitivity of these cell lines to MC-induced cytotoxicity. Compared to vehicle controls, treatment of HepG2 cells for 48 h with MC at 1 or 5 μ M decreased viability by 21% and 25%, respectively. Treatment of HepaRG cells for 48 h with MC at 1 or 5 μ M decreased viability by 32% and 46%, respectively.

Treatment of HepG2 cells with MC at 5 μ M caused marked induction of CYP1A1 mRNA levels (Fig. 2A), with a magnitude of 424-fold at 24 h and 247-fold at 48 h. The strong trends for induction by MC at 1 μ M at both time points did not achieve statistical significance. Although basal CYP2C8 mRNA levels in HepG2 cells were extremely low, we detected a 30% and 78% suppression of CYP2C8 mRNA levels at 24 h by the 1 and 5 μ M concentrations of MC, respectively (Fig. 2B). However, this suppression was transient with no apparent effects of MC on CYP2C8 mRNA levels persisting at 48 h in this cell line.

Treatment of HepaRG cells with MC at 1 μ M elicited 75- and 59-fold inductions of CYP1A1 mRNA levels at 24 and 48 h, respectively (Fig. 3A). MC at 5 μ M also produced marked induction of CYP1A1 mRNA levels, with magnitudes of 67- and 72-fold at 24 and 48 h, respectively. Although MC at 1 μ M did not alter CYP2C8 mRNA levels in HepaRG cells, MC at 5 μ M caused a pronounced 98% suppression of CYP2C8 mRNA levels at 48 h (Fig. 3B). The strong trend for suppression by the high MC concentration at 24 h did not achieve statistical significance. Compared to the response seen in HepG2 cells, the suppression of CYP2C8 mRNA levels by MC in HepaRG cells seems to be more pronounced and sustained. Additional experiments will be needed to explain this interesting difference between the cell lines, but we

speculate that the transient (HepG2) vs. sustained (HepaRG) *CYP2C8* suppression response may be related to cellular differences in MC detoxification, bioactivation, and/or cytotoxicity.

The relatively high basal *CYP2C8* expression, the robust *CYP1A1* induction, and the marked and sustained suppression of *CYP2C8* mRNA levels by MC are features that make HepaRG cells an attractive model for mechanistic studies. The HepaRG model is also suitable for studies of the functional consequences of *CYP2C8* down-regulation since *CYP2C8* catalytic activity has been measured in these cells (Antherieu et al. 2010), whereas *CYP2C8* protein is typically undetectable in HepG2 cells (Zhang et al. 2012). The more highly differentiated phenotype and higher metabolic activity of HepaRG cells likely means that MC is more rapidly metabolized in this cell line, possibly accounting for the lower magnitude of *CYP1A1* induction and greater cytotoxic sensitivity compared to HepG2 cells. A potential limitation of the HepaRG model could be the apparently narrow MC concentration range in which *CYP2C8* suppression can be studied. Although MC at both 1 and 5 μM causes similar, and likely maximal, AHR-mediated *CYP1A1* induction, the *CYP2C8* down-regulation response is absent at 1 μM and maximal at 5 μM . Cytotoxicity concerns preclude study of higher concentrations in this cell line.

If the AHR is involved in *CYP2C8* suppression, it would seem necessary to consider more complex and potentially indirect mechanisms compared to the AHR's canonical role in *CYP1A1* transcriptional induction. At the transcriptional level, mechanisms involving interference with *CYP2C8* regulation by HNF-4 α (Ferguson et al. 2005) could be explored. At the post-transcriptional level, miR-103 and miR-107 regulate *CYP2C8* expression, and increased levels of these miRNAs may be responsible for the low expression of *CYP2C8* protein in HepG2 cells compared to primary human hepatocytes (Zhang et al. 2012). Mechanistic roles for these factors and other players in the down-regulation of *CYP2C8* by PAHs should be explored in the terminally differentiated HepaRG model.

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References

- Antherieu, S., Chesne, C., Li, R., Camus, S., Lahoz, A., Picazo, L., et al. 2010. Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. *Drug Metab. Dispos.* **38**(3): 516-525. PMID: 20019244
- Attignou, E.A., Leblanc, A.F., Le-Grand, B., Duval, C., Aggerbeck, M., Rouach, H., et al. 2017. Novel roles for AhR and ARNT in the regulation of alcohol dehydrogenases in human hepatic cells. *Arch. Toxicol.* **91**(1): 313-324. PMID: 27055685
- Bhathena, A., Lee, C., and Riddick, D.S. 2002. Suppression of cytochrome P450C11 by aromatic hydrocarbons: mechanistic insights from studies of the 5'-flanking region of the *CYP2C11* gene. *Drug Metab. Dispos.* **30**(12): 1385-1392. PMID: 12433808
- Chen, Y., and Goldstein, J.A. 2009. The transcriptional regulation of the human *CYP2C* genes. *Curr. Drug Metab.* **10**(6): 567-578. PMID: 19702536
- Ferguson, S.S., Chen, Y.P., LeCluyse, E.L., Negishi, M., and Goldstein, J.A. 2005. Human *CYP2C8* is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4 α . *Mol. Pharmacol.* **68**(3): 747-757. PMID: 15933212

- Hart, S.N., Li, Y., Nakamoto, K., Subileau, E.A., Steen, D., and Zhong, X.B. 2010. A comparison of whole genome gene expression profiles of HepaRG cells and HepG2 cells to primary human hepatocytes and human liver tissues. *Drug Metab. Dispos.* **38**(6): 988-994. PMID: 20228232
- Jones, E.J., and Riddick, D.S. 1996. Regulation of constitutive rat hepatic cytochromes P450 by 3-methylcholanthrene. *Xenobiotica*, **26**(10): 995-1012. PMID: 8905915
- Lai, X.S., Yang, L.P., Li, X.T., Liu, J.P., Zhou, Z.W., and Zhou, S.F. 2009. Human CYP2C8: structure, substrate specificity, inhibitor selectivity, inducers and polymorphisms. *Curr. Drug Metab.* **10**(9): 1009-1047. PMID: 20214592
- Lee, C., and Riddick, D.S. 2000. Transcriptional suppression of cytochrome P450C11 gene expression by 3-methylcholanthrene. *Biochem. Pharmacol.* **59**(11): 1417-1423. PMID: 10751551
- Ning, B., Dial, S., Sun, Y., Wang, J., Yang, J., and Guo, L. 2008. Systematic and simultaneous gene profiling of 84 drug-metabolizing genes in primary human hepatocytes. *J. Biomol. Screen.* **13**(3): 194-201. PMID: 18270363
- Nishimura, M., Ueda, N., and Naito, S. 2003. Effects of dimethyl sulfoxide on the gene induction of cytochrome P450 isoforms, UGT-dependent glucuronosyl transferase isoforms, and ABCB1 in primary cultures of human hepatocytes. *Biol. Pharm. Bull.* **26**(7): 1052-1056. PMID: 12843640
- Park, S.H., and Waxman, D.J. 2001. Inhibitory cross-talk between STAT5b and liver nuclear factor HNF3 β : impact on the regulation of growth hormone pulse-stimulated, male-specific liver cytochrome P-450 gene expression. *J. Biol. Chem.* **276**(46): 43031-43039. PMID: 11562369

- Richert, L., Tuschl, G., Abadie, C., Blanchard, N., Pekthong, D., Manton, G., et al. 2009. Use of mRNA expression to detect the induction of drug metabolising enzymes in rat and human hepatocytes. *Toxicol. Appl. Pharmacol.* **235**(1): 86-96. PMID: 19118567
- Riddick, D.S., Lee, C., Bhathena, A., and Timsit, Y.E. 2003. The 2001 Veylien Henderson Award of the Society of Toxicology of Canada. Positive and negative transcriptional regulation of cytochromes P450 by polycyclic aromatic hydrocarbons. *Can. J. Physiol. Pharmacol.* **81**(1): 59-77. PMID: 12665258
- Roberts, E.A., Johnson, K.C., Harper, P.A., and Okey, A.B. 1990. Characterization of the Ah receptor mediating aryl hydrocarbon hydroxylase induction in the human liver cell line HepG2. *Arch. Biochem. Biophys.* **276**(2): 442-450. PMID: 2154949
- Safa, B., Lee, C., and Riddick, D.S. 1997. Role of the aromatic hydrocarbon receptor in the suppression of cytochrome P-450 2C11 by polycyclic aromatic hydrocarbons. *Toxicol. Lett.* **90**(2-3): 163-175. PMID: 9067484
- Sumida, A., Kinoshita, K., Fukuda, T., Matsuda, H., Yamamoto, I., Inaba, T., et al. 1999. Relationship between mRNA levels quantified by reverse transcription-competitive PCR and metabolic activity of CYP3A4 and CYP2E1 in human liver. *Biochem. Biophys. Res. Commun.* **262**(2): 499-503. PMID: 10462503
- Timsit, Y.E., and Riddick, D.S. 2000. Interference with growth hormone stimulation of hepatic cytochrome P450C11 expression in hypophysectomized male rats by 3-methylcholanthrene. *Toxicol. Appl. Pharmacol.* **163**(2): 105-114. PMID: 10698668
- Timsit, Y.E., and Riddick, D.S. 2002. Stimulation of hepatic signal transducer and activator of transcription 5b by GH is not altered by 3-methylcholanthrene. *Endocrinology*, **143**(9): 3284-3294. PMID: 12193540

Westerink, W.M., and Schoonen, W.G. 2007. Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells.

Toxicol. In Vitro, **21**(8): 1581-1591. PMID: 17637504

Zhang, S.Y., Surapureddi, S., Coulter, S., Ferguson, S.S., and Goldstein, J.A. 2012. Human CYP2C8 is post-transcriptionally regulated by microRNAs 103 and 107 in human liver.

Mol. Pharmacol. **82**(3): 529-540. PMID: 22723340

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Figure captions

Fig. 1. Comparison of CYP2C8 mRNA levels in human liver, HepaRG cells, and HepG2 cells. Quantitative analysis of CYP2C8 mRNA levels, relative to GAPDH. Values are the mean $\pm$ SD of determinations from three independent samples, expressed as a percentage of the mean for the human liver group. The individual data points for the three human liver RNA samples (K15, K16, K28) and three independent RNA isolations from HepaRG and HepG2 cultures are also shown. Data were analyzed by the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. * $P < 0.05$; significantly different from human liver group.

Fig. 2. Effect of MC treatment on CYP1A1 and CYP2C8 mRNA levels in HepG2 cells. Cells were treated with MC (1 or 5 μ M) or vehicle (0.5% DMSO) for 24 or 48 h. Quantitative analysis of (A) CYP1A1 and (B) CYP2C8 mRNA levels, relative to GAPDH. Values are the mean $\pm$ SD of three independent experiments, expressed as a percentage of the mean for the vehicle control group at each time point. Data were analyzed by randomized-design one-way ANOVA followed by Newman-Keuls multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; significantly different from time-matched vehicle control group.

Fig. 3. Effect of MC treatment on CYP1A1 and CYP2C8 mRNA levels in HepaRG cells. Cells were treated with MC (1 or 5 μ M) or vehicle (0.5% DMSO) for 24 or 48 h. Quantitative analysis of (A) CYP1A1 and (B) CYP2C8 mRNA levels, relative to GAPDH. Values are the mean $\pm$ SD of three independent experiments, expressed as a percentage of the mean for the vehicle control group at each time point. Data were analyzed by randomized-design one-way ANOVA followed by Newman-Keuls multiple comparisons test. * $P < 0.05$, ** $P < 0.01$; significantly different from time-matched vehicle control group.

Figure 1

Fig. 1

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Figure 2

Fig. 2

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Fig. 3

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