J|A|C|S COMMUNICATIONS

Published on Web 03/20/2009

Identification of an Overabundant Cholesterol Precursor in Hepatitis B Virus Replicating Cells by Untargeted Lipid Metabolite Profiling

Mary A. Rodgers,[†] Alan Saghatelian,[‡] and Priscilla L. Yang*,[†]

Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115, and Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

Received December 20, 2008; E-mail: priscilla_yang@hms.harvard.edu

Cellular lipids play essential but understudied roles in the replication of mammalian viruses. For example, the replication and assembly of many viruses occur on cellular membrane structures and the lipid bilayer of enveloped viruses is derived entirely from the host cell. Consequently, changes in lipid metabolism and signal transduction associated with viral infection and pathogenesis are of great interest as potential therapeutic targets for the treatment of viral diseases.[1−3] Significant advances in the use of quantitative mass spectrometry (MS) make this methodology ideal for interrogating virus-mediated alterations in host metabolism, as recently evidenced by the use of targeted MS methods to quantify changes in small molecule metabolites associated with cytomegalovirus infection.[4,5] Improvements in the detection and quantitative analysis of cellular lipids have, moreover, greatly increased the use of this methodology to characterize the lipid content of biological samples.[6,7] Recent advances in liquid chromatography−mass spectrometry (LC−MS) based untargeted metabolite profiling have enabled the identification of metabolites associated with specific disease states and would clearly complement existing virological methods that do not interrogate changes in low molecular weight metabolites. Toward this end, we have utilized LC−MS based untargeted metabolite profiling[8] to examine changes in steady-state levels of lipids in cells replicating human hepatitis B virus (HBV), a small enveloped virus that infects the liver, the organ primarily responsible for lipid metabolism.

Cell line HepG2.117[9] is a human hepatoma cell line engineered to stably replicate HBV in a tetracycline-controlled manner. Equal numbers of HBV-expressing (HBV+) and negative control (HBV−) cells from six independent cultures were homogenized in a 1:1:2 PBS−methanol−chloroform mixture, and total lipids in the chloroform phase were analyzed by positive mode and negative mode LC−MS. In all, over 10 000 individual mass ion intensities were compared between the HBV and control samples with the XCMS software package[10] (Figure 1).

The results from experiments performed in triplicate indicated that cells replicating HBV had small differences in the abundance of major lipid classes such as free fatty acids, phospholipids, and sphingolipids (Figure 1 and Supporting Information). These small fold changes may reflect significant changes in cellular composition in the case of highly abundant species. Notably, however, untargeted metabolite profiling indicated that HBV+ cells had a comparatively large overabundance of ion 367 *m*/*z* (M−OH) (Figure 1). Accurate mass calculations of this metabolite, **A**, indicate a formula of $C_{27}H_{44}O$, consistent with a dehydrogenated isomer of cholesterol. MS detection of **A** occurred at two elution times that presumably correspond to the free alcohol and ester forms. Notably, HBV

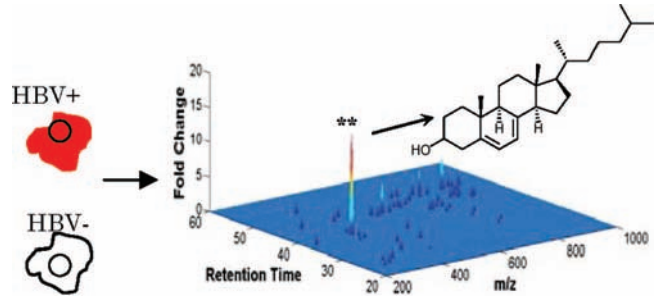

Figure 1. Untargeted lipid ion profiling of cells replicating HBV. Total lipids from HBV+ cells and negative control cells were quantified by LC−MS, and comparisons were made with XCMS software. ** indicates a *p*-value of less than 0.0003 by students *t* test.

replication was associated with a dose-dependent increase in the steady-state abundance of **A** (Supporting Information). Using a deuterated cholesterol standard for absolute quantification in picomoles, we discovered 18-fold and 4-fold enrichment of the free alcohol and ester forms of **A**, respectively, in HBV+ cells (Figure 2). For comparison, cholesterol esters were decreased by 2-fold

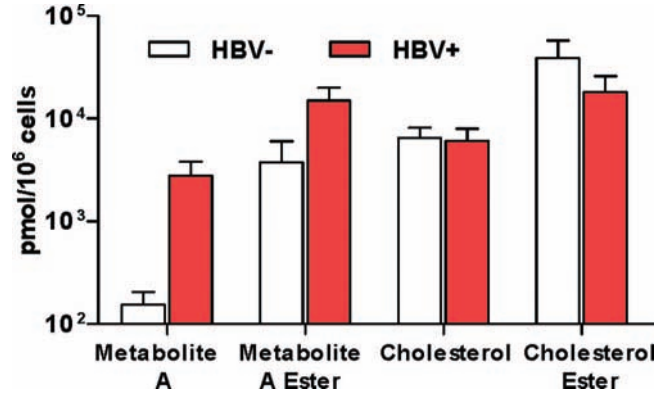

Figure 2. Quantification in biological triplicate of metabolite **A**, its ester, cholesterol, and cholesterol ester by normalization to deuterated cholesterol standard in HBV+ and HBV− cells.

and cholesterol was unchanged in HBV+ cells versus negative controls (Figure 2).

Four lines of evidence support that the structure of **A** is 7-dehydrocholesterol. First, the LC−MS retention time of **A** most closely matches that of a 7-dehydrocholesterol standard versus other standards of the same mass (Supporting Information). Second, coinjection of lipid extract from HBV+ cells with a 7-dehydrocholesterol standard resulted in a single peak for **A**, whereas coinjection of the HBV+ extract with other standards, such as desmosterol, resulted in two peaks (Supporting Information). Third,

[†] Harvard Medical School.
[‡] Harvard University.

10.1021/ja809949r CCC: $40.75

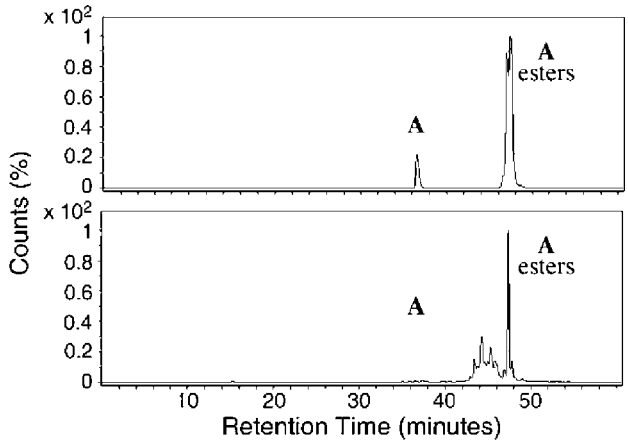

Figure 3. MS spectra for metabolite **A** from two aliquots of HBV+ total lipid extract. The peak corresponding to molecule **A** in HBV+ extracts (upper panel) disappears upon Diels−Alder reaction with 4-phenyl-1,2,4-triazoline-3,5-dione using a method previously reported for the selective derivatization of 7-DHC (lower panel).[11,12]

the peaks for **A** were consumed in a Diels−Alder reaction previously shown to specifically utilize 7-dehydrocholesterol[11,12] (Figure 3). Fourth, the ion fragmentation pattern of **A** matched that of the 7-dehydrocholesterol standard, but not the patterns of desmosterol, zymosterol, or vitamin D3 standards (Supporting Information). Collectively, these data unequivocally demonstrate that the identity of **A** is 7-dehydrocholesterol (7-DHC), the penultimate intermediate in cholesterol biosynthesis.

Sterols have increasingly been implicated as essential for the entry and fusion activity of many enveloped viruses.[13,14] Most previous studies have utilized cholesterol chelating agents or specific biosynthesis inhibitors,[15−19] both methods that deplete all intracellular sterols and that are therefore unable to resolve the role of individual sterols in the viral life cycle. Our results provide the first evidence that a virus is associated with accumulation of a specific lipid molecule, 7-DHC, and furthermore that this accumulation is selective versus all other cellular lipids detected by untargeted lipid metabolite profiling. The finding that viruses not only utilize cellular lipids but also have mechanisms for perturbing lipid metabolism highlights a growing appreciation of the mechanisms by which viruses exploit cellular resources to favor their own propagation. Coupled with increasing evidence from model systems that minor changes in sterol structure are associated with significant changes in functions including membrane dynamics,[20,21] lipid raft/domain formation,[22,23] and raft-associated receptor signaling,[24] our results provide strong motivation for ongoing studies characterizing the functional role of 7-DHC in HBV biology. Finally, our results illustrate the power of untargeted analytical methods in identifying metabolites that are critical in disease states, including viral infection and pathogenesis.

Acknowledgment. We thank Dr. James Cardia and Dr. Ranitendranath Tagore for helpful discussions and Dr. Michael Nassal for providing the HepG2.117 cell line. We gratefully acknowledge funding from The Alexander and Margaret Stewart Trust (P.L.Y.) and The Giovanni Armenise-Harvard Foundation (P.L.Y.), The Hellman Family Fund (P.L.Y.), and the Karnovsky Foundation (M.A.R). A.S. is a recipient of the Burroughs Wellcome Fund Career Award in the Biomedical Sciences and a Searle Scholars Award.

Supporting Information Available: The relative ion abundances of major lipid classes in HBV+ cells as well as molecule identification data and protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Raulin, J. *Prog. Lipid Res.* **2002**, *41*, 27–65.
(2) Barenholz, Y. *Subcell Biochem.* **2004**, *37*, 167–215.
(3) Ye, J. *PLoS Pathog.* **2007**, *3*, e108.
(4) Munger, J.; Bajad, S. U.; Coller, H. A.; Shenk, T.; Rabinowitz, J. D. *PLoS Pathog.* **2006**, *2*, e132.
(5) Munger, J.; Bennett, B. D.; Parikh, A.; Feng, X. J.; McArdle, J.; Rabitz, H. A.; Shenk, T.; Rabinowitz, J. D. *Nat. Biotechnol.* **2008**, *26*, 1179–86.
(6) Ivanova, P. T.; Milne, S. B.; Forrester, J. S.; Brown, H. A. *Mol. Interventions* **2004**, *4*, 86–96.
(7) Ivanova, P. T.; Milne, S. B.; Byrne, M. O.; Xiang, Y.; Brown, H. A. *Methods Enzymol.* **2007**, *432*, 21–57.
(8) Saghatelian, A.; Trauger, S. A.; Want, E. J.; Hawkins, E. G.; Siuzdak, G.; Cravatt, B. F. *Biochemistry* **2004**, *43*, 14332–9.
(9) Sun, D.; Nassal, M. *J. Hepatol.* **2006**, *45*, 636–45.
(10) Smith, C. A.; Want, E. J.; O'Maille, G.; Abagyan, R.; Siuzdak, G. *Anal. Chem.* **2006**, *78*, 779–87.
(11) Shimada, K.; Oe, T. *Anal. Sci.* **1990**, *6*, 461–463.
(12) Batta, A. K.; Salen, G.; Tint, G. S.; Honda, A.; Shefer, S. *Steroids* **1997**, *62*, 700–2.
(13) Rawat, S. S.; Viard, M.; Gallo, S. A.; Rein, A.; Blumenthal, R.; Puri, A. *Mol. Membr. Biol.* **2003**, *20*, 243–54.
(14) Teissier, E.; Pecheur, E. I. *Eur. Biophys. J.* **2007**, *36*, 887–99.
(15) Finnegan, C. M.; Rawat, S. S.; Puri, A.; Wang, J. M.; Ruscetti, F. W.; Blumenthal, R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15452–7.
(16) Ikeda, M.; Abe, K.; Yamada, M.; Dansako, H.; Naka, K.; Kato, N. *Hepatology* **2006**, *44*, 117–25.
(17) Kapadia, S. B.; Barth, H.; Baumert, T.; McKeating, J. A.; Chisari, F. V. *J. Virol.* **2007**, *81*, 374–83.
(18) Funk, A.; Mhamdi, M.; Hohenberg, H.; Heeren, J.; Reimer, R.; Lambert, C.; Prange, R.; Sirma, H. *J. Virol.* **2008**, *82*, 10532–42.
(19) Bremer, C. M.; Bung, C.; Kott, N.; Hardt, M.; Glebe, D. *Cell Microbiol* **2008**, *11*, 249–60.
(20) Cao, H.; Tokutake, N.; Regen, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 16182–3.
(21) Shrivastava, S.; Paila, Y. D.; Dutta, A.; Chattopadhyay, A. *Biochemistry* **2008**, *47*, 5668–77.
(22) Xu, X.; London, E. *Biochemistry* **2000**, *39*, 843–9.
(23) Megha, Bakht, O.; London, E. *J. Biol. Chem.* **2006**, *281*, 21903–13.
(24) Pucadyil, T. J.; Shrivastava, S.; Chattopadhyay, A. *Biochem. Biophys. Res. Commun.* **2005**, *331*, 422–7.

JA809949R