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Cholesterol biosynthesis regulation and protein changes in rat liver following treatment with fluvastatin

Sandra Steiner *, Christine L. Gatlin, John J. Lennon, Andrew M. McGrath, Michael D. Seonarain, Anthony J. Makusky, Angel M. Aponte, Ricardo Esquer-Blasco, N. Leigh Anderson

Large Scale Proteomics Corporation, 9620 Medical Center Drive, Suite 201, Rockville, MD 20850, USA

Abstract

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is a key regulator in cholesterol biosynthesis and HMG CoA reductase inhibitors (statins) have become a widely prescribed family of lipid lowering agents. Cholesterol synthesis occurs predominantly in liver which is the target organ of statins. We studied the effects of fluvastatin (Lescol®), a member of the statin family, on hepatic protein regulation. Male F344 rats treated with 0.8 mg/kg per day fluvastatin or 24 mg/kg per day fluvastatin for 7 days showed treatment-related changes in 58 liver proteins ($P < 0.005$). Major effects were evident in the cholesterol biosynthesis pathway including the induction of enzymes upstream and downstream of the target enzyme HMG CoA reductase. Treatment also triggered alterations in key enzymes of carbohydrate metabolism and was associated with changes in a heterogeneous set of cellular stress proteins involved in cytoskeletal structure, calcium homeostasis and protease activity. The latter set of protein alterations indicates that hepatotoxicity is associated with high-dose treatment. Based on the results it is suggested that HMG-CoA synthase and isopentenyl-diphosphate delta-isomerase may be explored as alternative drug targets and that the induction levels of these enzymes may serve as a measure of potency of individual statin drugs. It is proposed that efficacy and cellular stress markers discovered in this study may be used in a high throughput screen (HTS) assay format to compare efficiently and accurately the therapeutic windows of different members of the statin family.

Keywords: Fluvastatin; Proteomics; Rat liver; Two-dimensional electrophoresis; Mass spectrometry; Pathway regulation

1. Introduction

High levels of low-density lipoprotein (LDL) cholesterol and low levels of high-density lipoprotein (HDL) cholesterol are both known risk factors for coronary heart disease. In addition, the involvement of LDL cholesterol in atherogenesis has been well documented in clinical studies. Cholesterol is synthesized predominantly in the liver and transported to various body tissues by lipoproteins in blood plasma. Therapeutic interventions to normalize elevated plasma LDL

* Corresponding author: Tel.: +1-301-4245989; fax: +1-301-7624892.

E-mail address: sandra.steiner@lsp.lsbc.com (S. Steiner).

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cholesterol levels in hypercholesterolemic individuals are in widespread use. Of these, HMG CoA reductase inhibitors have become the most widely prescribed family of agents (such as fluvastatin, pravastatin, simvastatin, lovastatin, atorvastatin). HMG-CoA reductase is the key regulatory enzyme in the biosynthetic pathway for cholesterol and catalyzes the conversion of HMG-CoA to mevalonate. The inhibition of this enzyme results in both the down-regulation of cholesterol synthesis and the up-regulation of hepatic high affinity receptors for low density lipoproteins (LDL) followed by increased catabolism of LDL cholesterol (Goldstein and Brown, 1984). Otherwise, HMG-CoA reductase inhibitors do not affect, to a significant extent, the levels and/or composition of the other major lipoprotein fractions (Sirtori, 1990).

Fluvastatin is the first entirely synthetic HMG-CoA reductase inhibitor, and is in part structurally distinct from the fungal derivatives of this therapeutic class, and is sold under the brand name Lescol® in the United States. Fluvastatin appears in much higher concentrations in the liver than in non-target organs, and the liver is the drug's primary site of both action and side effects. Marked increases in serum transaminases and biochemical abnormalities of liver function occurred in a subset of patients who received fluvastatin or other HMG-CoA reductase inhibitors for an extended period of time (Swislocki et al., 1997).

It is straightforward to imagine that the therapeutic inhibition of a key enzyme of the cholesterol synthesis pathway is not silently tolerated by the organism but evokes a response targeted to compensate for disturbed pathway performance. Such a regulatory feedback effect may contribute to the pharmacological action of a drug, e.g. the up-regulation of LDL receptors (Goldstein and Brown, 1990), but equally is often associated with adverse reactions. The purpose of this study was to gain molecular insights into the liver effects induced by fluvastatin and to elucidate the biochemical pathways and gene network regulations induced downstream of the blockade of HMG-CoA reductase. Tissue proteome analysis has been successfully applied (Cunningham et al., 1995; Myers et al., 1995; Anderson et al., 1996a,b; Steiner et al., 1996; Aicher et al., 1998; Arce et al., 1998) to investigate the molecular effects of drugs and to obtain information on their mode of action and mechanisms of toxicity. In this case, we used proteomics to study proteome changes in livers of rats treated for 7 days with either 0.8 or 24 mg/kg per day fluvastatin. The drug was found to induce a complex pattern of alterations in rat liver proteins, some of which were related to cholesterol synthesis but many were representing effects on other pathways and endpoints.

2. Material and methods

2.1. Reagents

Ultrapure reagents for polyacrylamide gel preparation were obtained from Bio-Rad (Richmond, CA). Ampholytes pH 4–8 were from BDH (Poole, UK), ampholytes pH 8–10.5 were from Pharmacia (Uppsala, Sweden) and CHAPS was obtained from Calbiochem (La Jolla, CA). Deionized water from a high purity water system (NeuIon, Inc., Baltimore, MD) was used. System filters were changed monthly to ensure 18 MΩ purity. HPLC grade methanol and glacial acetic acid were furnished from Fisher Scientific (Fair Lawn, NJ). HPLC grade acetonitrile was obtained from Baker (Phillipsburg, NJ). Dithiothreitol (DTT) was obtained from Gallard-Schlesinger Industries (Carle Place, NY). Iodoacetamide, ammonium bicarbonate, trifluoroacetic acid and α-cyano-4-hydroxycinnamic acid were obtained from Sigma (St Louis, MO). Modified porcine trypsin was purchased from Promega (Madison, WI). All chemicals (unless specified) were reagent grade and used without further purification.

2.2. Animal treatment protocol

Male F344 rats (Charles River, Raleigh, NC) 8 weeks of age and weighing 148–176 g were used. The animals were housed individually in rat gang cages in an environmentally controlled room and were fed with Rodent Chow (Research Diets Inc., New Brunswick, NJ) and tap water ad libitum.

Three groups of five rats each received control feed, rodent chow milled with 8 ppm (approximately 0.8 mg/kg per day) fluvastatin and rodent chow milled with 240 ppm (approximately 24 mg/kg per day) fluvastatin for 7 days. The animals were guillotined after CO_2 asphyxiation 1 day following the last treatment. Liver samples (150 mg of the left apical lobe) were removed and flash frozen in liquid nitrogen and kept at −80°C until analysis.

2.3. Sample preparation

The samples were homogenized in 8 vol. of 9 M urea, 2% CHAPS, 0.5% dithiothreitol (DTT) and 2% carrier ampholytes pH 8–10.5. The homogenates were centrifuged at 420 000 × *g* at 22°C for 30 min (TL100 ultracentrifuge, TLA 100.3 rotor, 100 000 rev./min; Beckman Instruments, Palo Alto, CA). The supernatant was removed, divided into four aliquots and stored at −80°C until analysis.

2.4. Two-dimensional gel electrophoresis

Sample proteins were resolved using the 20 × 25 cm ISO-DALT® 2-D system (Anderson et al., 1991). Solubilized samples (8 μl) were applied to each gel and the gels were run for 25 050 V h using a progressively increasing voltage with a high-voltage programmable power supply. An Angelique™ computer-controlled gradient-casting system (Large Scale Proteomics Corporation, Rockville, MD) was used to prepare the second-dimension SDS slab gels. The top 5% of each gel was 11% T acrylamide and the lower 95% of the gel varied linearly from 11 to 19% T. The IEF gels were loaded directly on to the slab gels using an equilibration buffer with a blue tracking dye and were held in place with a 1% agarose overlay. Second-dimensional slab gels were run overnight at 160 V in cooled DALT tanks (10°C) with buffer circulation and were taken out when the tracking dye reached the bottom of the gel. Following SDS electrophoresis, the slab gels were fixed overnight in 1.5 l/10 gels of 50% ethanol/3% phosphoric acid and then washed three times for 30 min in 1.5 l/10 gels of cold DI water. They were transferred to 1.5 l/10 gels of 34% methanol/17% ammonium sulfate/3% phosphoric acid for 1 h and, after the addition of 1 g powdered Coomassie Blue G-250, the gels were stained for 3 days to achieve equilibrium intensity.

2.5. Quantitative gel pattern analysis

Stained slab gels were digitized in red light at 133-μm resolution, using an Eikonix 1412 scanner and images were processed using the Kepler® software system as described (Shevchenko et al., 1996). Groupwise statistical comparisons were made to search for treatment-related protein abundance changes.

2.6. Protein digestion

Gel pieces containing the proteins of interest were excised from a Coomassie stained gel and placed in a 96-well polypropylene microtiter plate. Samples were digested in-gel with trypsin according to the procedure of Shevchenko et al. (1996) with slight modifications. Briefly, the excised samples were destained by two 60-min cycles of bath sonication in 0.2 M NH_4HCO_3 in 50% CH_3CN with the resulting solution aspirated after each cycle. A volume of 0.2 M NH_4HCO_3 in 50% CH_3CN to cover the gel pieces sufficiently was added. Reduction and alkylation were accomplished by adding 135 nmol DTT and incubating at 37°C for 20 min. After cooling, 400 nmol of iodoacetamide was added and incubated at room temperature in the dark for 20 min. The supernatant was removed and the samples were washed for 15 min in 0.2 M NH_4HCO_3 in 50% CH_3CN. The gel pieces were dried at 37°C for 15 min and partially rehydrated with 5 μl 0.2 M NH_4HCO_3. After dispensing 3 μl of trypsin (30 ng/μl), the samples were incubated at room temperature for 5 min. A sufficient volume of 0.2 M NH_4HCO_3 was added to ensure complete submersion of the gel pieces in the digestion buffer. Samples were incubated overnight at 37°C. All samples were acidified with 1 μl glacial acetic acid. Tryptic peptides were extracted by initially transferring the digest supernatant to a clean 96-well polypropylene microtiter plate with two subse-

quent extraction and transfer cycles of 60 μl of 60% CH_3CN, 1% glacial acetic acid. The combined extraction supernatant was dried and reconstituted in 6 μl 1% glacial acetic acid for subsequent mass spectral analysis.

2.7. Matrix-assisted laser desorption mass spectrometric (MALDI) protein identification

All samples were prepared using α-cyano-4-hydroxycinnamic acid as the MALDI matrix using the dried droplet method (Karas and Hillenkamp, 1988). The matrix solution was saturated in 40% CH_3CN, 0.1% trifluoroacetic acid in water. The peptide solution (1.0 μl) was applied first to the smooth, sample plate target, then 1.0 μl of matrix solution was stirred in with a pipette tip and the sample allowed to air evaporate.

MALDI experiments were performed on a PerSeptive Biosystems Voyager-DE STR time-of-flight mass spectrometer (2.0 m linear flight path) equipped with delayed ion extraction. A pulsed nitrogen laser (Model VSL-337ND, Laser Science, Franklin, MA) at 337.1 nm (<4 ns FWHM pulse width) was used for all of the data acquisition. Data were acquired in the delayed ion extraction mode using a 20-kV bias potential, a 6-kV pulse and a 150-ns pulsed delay time. Dual microchannel plate (Model 3040MA, Burle Electro-Optics, Sturbridge, MA) detection was used in the reflector mode with the ion signal recorded using a 2-GHz transient digitizer (Model TDS 540C, Wilsonville, OR) at a rate of 1 GS/s. All mass spectra represent signal averaging of 128 laser pulses. The performance of the mass spectrometer produced sufficient mass resolution to produce the isotopic multiplet for each ion species below mass-to-charge (m/z) of 3000. The data were analyzed using GRAMS/386 software (Galactic Industries, Salem, NH).

All MALDI mass spectra were internally calibrated using masses from two trypsin autolysis products (monoisotopic masses 841.50 and 2210.10). Mass spectral peaks were determined based on a signal-to-noise (S/N) ratio of 3. Two software packages, Protein Prospector and Profound, were used to identify protein spots. The rat and mouse nonredundant (NR) database consisting of SwissProt, PIR, GeneBank and OWL was used in the searches. Parameters used in the searches included proteins less than 100 kDa, greater than four matching peptides and mass errors less than 45 ppm.

2.8. Liquid chromatography-tandem mass spectrometry protein identification

A home-built microelectrospray interface similar to an interface described by Gatlin et al. (1998) was employed. Briefly, the interface uses a PEEK micro-tee (Upchurch Scientific, Oak Harbor, WA) into one stem of which is inserted a 0.025″ gold wire to supply the electrical connection. Spray voltage was 1.8 kV. A microcapillary column was prepared by packing 10 μm MAGIC C18 particles (Michrom BioResources, Auburn, CA) to a depth of 10 cm into a 75 × 360 μm fused silica capillary PicoTip (New Objectives, Cambridge, MA). A 50–70 μl/min flow from a MAGIC 2002 HPLC solvent delivery system (Michrom BioResources) was reduced using a splitting tee to achieve a column flow rate of 350–450 nl/min.

Samples were loaded on-column using an Alcott model 718 autosampler (Alcott Chromatography, Norcross, GA). HPLC flow was split prior to sample loop injection. Samples prepared for MALDI were diluted 1:3 in 0.5% HOAc, and 2 μl of each sample was injected on-column. Using contact closures, the HPLC triggered the autosampler to make an injection and after a set delay time, triggered the mass spectrometer to start data collection.

A 12-min gradient of 5–55% solvent B (A: 2% ACN/0.5% HOAc, B: 90% ACN/0.5% HOAc) was selected for separation of trypsin digested peptides. Peptide analyses were performed on a Finnigan LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA). The heated desolvation capillary was set at 150°C, and the electron multiplier at −900 V. Spectra were acquired in automated MS/MS mode with a relative collision energy (RCE) preset to 35%. To maximize data acquisition efficiency, the additional parameters of dynamic exclusion, isotopic exclusion and 'top 3 ions' were incorporated into the auto-MS/MS

procedure. For the 'top 3 ions' parameter, an MS spectrum was taken followed by three MS/MS spectra corresponding to the three most abundant ions above threshold in the full scan. This cycle was repeated throughout the acquisition. The scan range for MS mode was set at m/z 375–1200. A parent ion default charge state of $+2$ was used to calculate the scan range for acquiring tandem MS.

Automated analysis of LCQ peptide tandem mass spectra was performed using the computer algorithms SEQUEST (Finnigan MAT, San Jose, CA) and/or Mascot (Matrix Science Ltd, London, UK). The non-redundant (NR) protein database was obtained as an ASCII text file in FASTA format from the National Center for Biotechnology Information (NCBI). A specific rat protein database was created by selecting rat protein sequences from the NR database. This database subset was used for subsequent searches. Protein identifications were based on obtaining good quality MS/MS spectra from a minimum of two unique tryptic peptides.

3. Results and discussion

All animals tolerated and survived the treatment, however a decrease in food consumption in the high dose group occurred resulting in a decrease in weight gain for this group. Over the 7-day treatment period, the average weight gain was 43.5 g in control animals, 37.6 g in the low dose and 5.4 g in the high dose treatment group. The 2-DE protein pattern of an F344 rat liver shows over 1000 Coomassie Blue-stained protein spots. Fluvastatin treatment altered the abundance of 55 liver spots, based on the application of the two-tailed Student's t-test ($P < 0.005$). Most of the statistically significant changes occurred in the group receiving 240 ppm fluvastatin in feed for 7 days, a concentration similar to the high dose used in the 24-month carcinogenicity study in rats (Physicians' Desk Reference, 2000). Changes were evident in livers of rats treated with 8 ppm fluvastatin for 7 days, an exposure comparable to the maximum recommended daily dose in humans, but did not reach as high a level of statistical significance. The proteins affected by the treatment with high significance ($P < 0.005$) are listed in Table 1. The changes induced by fluvastatin were similar to ones found with lovastatin, another member of the HMG-CoA reductase inhibitor drug family that was investigated by us (Steiner et al., 2000). Many of the affected proteins were already identified in the F344 rat liver reference 2-D pattern (Anderson et al., 1995; Steiner et al., 2000). Several spots not yet identified but significantly altered by fluvastatin treatment were subjected to tryptic-digestion and MALDI-MS and LC-MS/MS analysis and were unambiguously identified by this approach.

3.1. Cholesterol synthesis pathway

Fluvastatin treatment showed a profound effect on cholesterol biosynthesis. Several enzymes located downstream and upstream of the drug target HMG-CoA reductase were induced in this pathway. Both cytosolic and mitochondrial HMG-CoA synthase, two enzymes with similar functions but encoded by different genes (Ayte et al., 1990) were increased several-fold by the treatment. While cytosolic HMG-CoA synthase is involved in cholesterol biosynthesis, mitochondrial HMG-CoA synthase is part of the ketone body synthesis pathway and was found greatly increased by starvation, fat feeding and diabetes (Casals et al., 1992). Isopentenyl-diphosphate delta-isomerase (IPP-isomerase), an enzyme participating in the cholesterol biosynthesis pathway downstream of HMG-CoA reductase showed the most prominent treatment effect and was increased more than 24-fold in the high dose group. The data suggest that the up-regulation of the cholesterol synthesis pathway is a feedback reaction to the blockade of HMG-CoA reductase and an attempt of the liver to compensate for the impaired pathway performance. The degree to which these enzymes are induced seems to be linked to the degree to which HMG-CoA reductase is inhibited and thus serves as a measure of the pharmacological potency of the drug. Hence, it is possible to use, for example, HMG-CoA synthase and IPP-isomerase induction levels to compare the relative potencies between different members of the statin drug family.

Table 1
Changes in the rat liver proteome following treatment with fluvastatin for 7 days[a]

MSN	Protein name	Control		Fluvastatin 1.6 mg/kg per day					Fluvastatin 150 mg/kg per day				
		Avol	CV	Avol	CV	Prob	Ratio	N-fold	Avol	CV	Prob	Ratio	N-fold
29	Keratin, type I cytoskeletal 18	31 578	0.532	36 511	0.124	0.59537	1.16	1.16	83 115	0.195	0.00271	2.63	2.63
34		20 327	0.125	22 765	0.104	0.17944	1.12	1.12	9673	0.090	0.00051	0.48	2.10
41	Keratin, type II cytoskeletal 8	32 650	0.161	37 033	0.270	0.53798	1.13	1.13	88 299	0.180	0.00056	2.70	2.70
55	Senescence marker protein-30	56 130	0.174	45 287	0.373	0.29486	0.81	1.24	19 338	0.133	0.00024	0.34	2.90
59	Aldehyde dehydrogenase, mitochondrial (EC 1.2.1.3)	11 086	0.148	13 725	0.288	0.26299	1.24	1.24	4002	0.133	0.00149	0.36	2.77
66		9330	0.091	8608	0.230	0.52784	0.92	1.08	3223	0.269	0.00010	0.35	2.89
69		13 526	0.071	13 121	0.081	0.57829	0.97	1.03	8090	0.140	0.00032	0.60	1.67
76		12 165	0.046	11 722	0.184	0.70348	0.96	1.04	7810	0.201	0.00161	0.64	1.56
79	Fructose-1,6-bisphosphatase (EC 3.1.3.11)	18 087	0.068	17 317	0.111	0.51674	0.96	1.04	6933	0.153	0.00004	0.38	2.61
83		17 158	0.043	15 844	0.246	0.53811	0.92	1.08	13 181	0.113	0.00230	0.77	1.30
91	Isovaleryl-CoA Dehydrogenase (EC 1.3.99.10)	13 307	0.072	14 781	0.344	0.59514	1.11	1.11	8660	0.141	0.00074	0.65	1.54
97	Keratin, type II cytoskeletal 8	14 515	0.096	14 571	0.310	0.97990	1.00	1.00	40 496	0.115	0.00010	2.79	2.79
101	*S*-Adenosylmethionine synthase (EC 2.5.1.6)	15 614	0.125	13 726	0.198	0.28244	0.88	1.14	7562	0.375	0.00234	0.48	2.06
103	Senescence marker protein-30	5946	0.127	10 493	0.076	0.00044	1.76	1.76	1501	0.252	0.00021	0.25	3.96
113	Adenosine kinase (EC 2.7.1.20)	17 125	0.022	15 974	0.135	0.33414	0.93	1.07	11 289	0.043	0.00002	0.66	1.52
117	Aryl sulfotransferase (EC 2.8.2.1)	29 811	0.159	27 542	0.137	0.54579	0.92	1.08	18 447	0.113	0.00223	0.62	1.62
125	*N*-hydroxyarylamine sulfotransferase (EC 2.8.2.-)	26 941	0.081	27 941	0.134	0.65424	1.04	1.04	11 648	0.224	0.00015	0.43	2.31
126	3-Hydroxyanthranilate 3,4-dioxygenase (EC 1.13.11.6)	10 811	0.098	9757	0.320	0.54832	0.90	1.11	4273	0.123	0.00006	0.40	2.53
127		12 246	0.033	10 681	0.084	0.01491	0.87	1.15	9503	0.104	0.00168	0.78	1.29
128		3173	0.159	3367	0.438	0.80554	1.06	1.06	721	0.458	0.00018	0.23	4.40
142	Ketohexokinase (EC 2.7.1.3)	20 651	0.191	19 106	0.244	0.61793	0.93	1.08	9889	0.201	0.00142	0.48	2.09
143		3674	0.113	3776	0.230	0.83131	1.03	1.03	2117	0.195	0.00118	0.58	1.74
155		5405	0.297	4981	0.210	0.64874	0.92	1.09	2032	0.261	0.00331	0.38	2.66
162	D-Dopachrome tautomerase (EC 5.3.3.12)	26 432	0.212	20 743	0.311	0.20597	0.78	1.27	12 183	0.349	0.00374	0.46	2.17
168	Antiquitin-rat (fragment)	12 634	0.067	11 607	0.096	0.16957	0.92	1.09	7586	0.156	0.00041	0.60	1.67
172	Tropomyosin	7474	0.095	7101	0.239	0.69643	0.95	1.05	10 169	0.094	0.00271	1.36	1.36
178		7866	0.036	8528	0.081	0.11616	1.08	1.08	4995	0.095	0.00010	0.64	1.57
182	Fructose1,6-bisphosphatase (EC 3.1.3.11)	7352	0.086	6704	0.275	0.53336	0.91	1.10	1908	0.203	0.00003	0.26	3.85
252	Contrapsin-like protease inhibitor 1	4769	0.086	3807	0.313	0.16936	0.80	1.25	2105	0.323	0.00195	0.44	2.27
289		6659	0.083	5970	0.259	0.56673	0.90	1.12	3207	0.258	0.00042	0.48	2.08
307		8067	0.083	8005	0.171	0.93487	0.99	1.01	4579	0.174	0.00045	0.57	1.76
311		9521	0.559	12 836	0.134	0.22456	1.35	1.35	25 424	0.145	0.00149	2.67	2.67
315	Pyruvate kinase, isozymes R/L (EC 2.7.1.40)	7792	0.087	7721	0.190	0.92893	0.99	1.01	3506	0.273	0.00033	0.45	2.22
318		5961	0.074	5410	0.140	0.24076	0.91	1.10	2798	0.290	0.00046	0.47	2.13
358		2926	0.068	2772	0.180	0.58940	0.95	1.06	1868	0.136	0.00051	0.64	1.57
361	Hydroxymethylglutaryl-CoA synthase, mitoch. frag.	6857	0.515	8534	0.192	0.62527	1.24	1.24	26 398	0.131	0.00022	3.85	3.85
362		4650	0.078	5375	0.213	0.26702	1.16	1.16	1742	0.190	0.00015	0.37	2.67
371		6782	0.159	6390	0.129	0.58463	0.94	1.06	2978	0.381	0.00394	0.44	2.28
372		9962	0.287	10 847	0.058	0.62998	1.09	1.09	62 658	0.193	0.00139	6.29	6.29
413	Hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5)	5738	0.075	8989	0.104	0.00067	1.57	1.57	30 440	0.184	0.00020	5.31	5.31
420		3714	0.085	3928	0.109	0.56097	1.06	1.06	1191	0.400	0.00242	0.32	3.12
492		3121	0.166	2846	0.260	0.55556	0.91	1.10	1530	0.249	0.00148	0.49	2.04
501	2-Oxoisovalerate dehydrogenase alpha subunit, mitoch.	4386	0.087	4499	0.093	0.69001	1.03	1.03	929	0.368	0.00011	0.21	4.72
506		4431	0.141	4528	0.099	0.80324	1.02	1.02	2715	0.121	0.00147	0.61	1.63
590	Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	949	0.083	1312	0.189	0.02610	1.38	1.38	2434	0.203	0.00096	2.57	2.57
613		1279	0.100	1353	0.117	0.50349	1.06	1.06	752	0.198	0.00221	0.59	1.70
644		2846	0.103	2356	0.258	0.18327	0.83	1.21	1212	0.494	0.00205	0.43	2.35
669		1441	0.069	1242	0.268	0.28981	0.86	1.16	682	0.187	0.00013	0.47	2.11
681		4402	0.171	3880	0.289	0.54199	0.88	1.13	1244	0.495	0.00046	0.28	3.54
721		477	0.100	210	0.208	0.00355	0.44	2.27	396	NA	NA	0.83	1.21
802		1258	0.115	1165	0.200	0.52725	0.93	1.08	741	0.172	0.00222	0.59	1.70
900		1101	0.009	451	0.231	0.00223	0.41	2.44	848	0.479	0.54340	0.77	1.30
934	ras-GTPase-activating protein SH3-domain domain binding protein	810	0.146	474	0.186	0.00435	0.59	1.71	416	0.541	0.01622	0.51	1.94
1119	Isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2)	632	0.098	1451	0.436	0.14378	2.29	2.29	15 381	0.065	0.00014	24.32	24.32
1250	Hydroxymethylglutaryl-coenzyme A synthase	687	0.236	1735	0.278	0.00485	2.53	2.53	2163	0.277	0.00546	3.15	3.15

[a] MSN, the F344MST3 liver master spot number; Avol., the group average optical density value of a protein spot; CV, coefficient of variation; Prob, the *P*-value when compared to the control group (Student's *t*-test). Ratio, the ratio of change relative to the control; N-fold, the magnitude of change.

3.2. Lipid metabolism

Apolipoprotein A-I was induced more than twofold in the high dose treatment group ($P < 0.009$). Apolipoprotein A-I is synthesized in the liver and secreted into the blood, and is the major protein of plasma HDL and also found in chylomicrons. HDL mediates the reverse transport of cholesterol from tissues to the liver, the site of cholesterol metabolism and secretion. Thus, the increased abundance of precursor apolipoprotein A-I suggests an up-regulation of HDL synthesis coupled with an increase in cholesterol catabolism in the liver. As discussed earlier, liver LDL receptors are expected to be up-regulated following fluvastatin treatment (Goldstein and Brown, 1984; Arce et al., 1998). The LDL receptor is a membrane protein that likely is not solubilized under the conditions used in this protocol and hence is missing in this data set. Decreases were found in mitochondrial aldehyde dehydrogenase, involved in numerous biochemical pathways including fatty acid metabolism and bile acid biosynthesis and antiquitin, a member of the superfamily of aldehyde dehydrogenases.

3.3. Carbohydrate metabolism

Protein regulations induced by fluvastatin were not restricted to the intentionally targeted cholesterol synthesis pathway but also included the interlinked carbohydrate metabolism. Several enzymes in this pathway were down-regulated by the treatment such as fructose-1,6-bisphosphatase, a key regulatory enzyme necessary for, and specific to, gluconeogenesis or ketohexokinase, involved in metabolism of fructose or pyruvate kinase, involved in the final step in glycolysis. Deficiency of fructose-1,6-bisphosphatase is associated with fasting hypoglycemia and metabolic acidosis because of impaired gluconeogenesis (el-Maghrabi et al., 1995). An increase was found in glucose-6-phosphate 1-dehydrogenase, the first enzyme in the pentose phosphate pathway suggesting a drug-induced up-regulation of this pathway. Some of these alterations may be related to the decrease in weight gain that occurred in the high dose treatment group, emphasizing the importance of the confounding influence of food intake in the interpretation of the data.

3.4. Cellular stress

Several proteins associated with cellular stress were affected by fluvastatin treatment. Senescence marker protein-30 (SMP-30), a cytosolic protein with decreased expression during senescent stages, and recently reported to be identical to a calcium binding protein called regucalcin (Fujita et al., 1999) showed a treatment-related decrease. SMP30 regulates calcium homeostasis by enhancing plasma membrane calcium-pumping activity (Fujita et al., 1998) and its down-regulation suggests deregulation of calcium signaling and cellular stress. Contrapsin-like protease inhibitor 1 (serine protease inhibitor 2), a protein found to be down-regulated in inflammation, was decreased by the treatment suggesting high doses of fluvastatin were associated with inflammatory processes in liver. Keratin type I cytoskeletal 18 and keratin type II cytoskeletal 8, the two components of the intermediate filament family were equally induced upon fluvastatin treatment. Cytokeratin filaments are important components of the cytoskeletal structure and their alterations may reflect cellular stress. A slight induction was also observed in tropomyosin, a protein found in the thin filaments of muscle fibers. Changes in these sets of proteins are likely indicators of liver toxicity induced following high dose fluvastatin treatment. Hence, the degree to which these proteins are altered by specific drugs may serve as a measure of toxicity associated with the treatment.

3.5. Nucleotide and amino acid metabolism

Several proteins involved in nucleotide and amino acid metabolism were down-regulated by the treatment such as adenosine kinase, the key intracellular enzyme regulating intra- and extracellular adenosine concentrations. Inhibition of adenosine kinase is known to produce marked increases in extracellular adenosine levels that are localized to cells and tissues undergoing accelerated adenosine release (Kowaluk et al., 1998). Similar decreases were evident in 3-hydroxyan-

thranilate 3,4-dioxygenase, *S*-adenosylmethionine synthase, aminoacylase-1 and 2-oxoisovalerate dehydrogenase.

In addition, two sulfotransferases, *N*-hydroxyarylamine sulfotransferase, a liver specific enzyme and aryl sulfotransferase were decreased by fluvastatin.

4. Conclusions

Proteome analysis revealed quantitative changes in a high number of rat hepatic proteins following 7-day treatment with the lipid lowering agent and marketed pharmaceutical, fluvastatin (Lescol®). Major protein regulations were evident in the cholesterol synthesis pathway including the induction of enzymes upstream and downstream of the target enzyme HMG-CoA reductase, demonstrating the rigorous attempt of the liver to counteract the drug and to maintain normal cholesterol synthesis rates. Based on these data we suggest that HMG-CoA synthase and IPP-isomerase may be explored as alternative drug targets to control cholesterol synthesis. In fact, reports are available documenting that HMG-CoA synthase has been evaluated as a target for therapeutic intervention (Greenspan et al., 1987). Attempts to target IPP-isomerase therapeutically have, to our knowledge, not been reported in the public domain. In addition it is suggested that the induction levels of the above enzymes may serve as a measure to determine the potency of individual statin drugs. Fluvastatin treatment also triggered alterations in key enzymes of carbohydrate metabolism, and was associated with changes in a heterogeneous set of cellular stress proteins involved in cytoskeletal structure, calcium homeostasis and protease activity. It is suggested that the degree to which cellular stress proteins are changed upon treatment may serve as a measure of toxicity induced by the drug. In essence, this work demonstrates the power of proteomics to obtain insights into the molecular effects of drugs and to discover new drug targets or protein markers of drug efficacy and toxicity. Efficacy and toxicity protein markers selected in such studies may be used in HTS assay formats for various applications in drug discovery and development. The combination of efficacy and toxicity measurements in one test system may, for example, represent an efficient and accurate way to determine the therapeutic window of a drug and to compare different members of compound families in order to prioritize the most promising ones for development.

References

Aicher, L., Wahl, D., Arce, A., Grenet, O., Steiner, S., 1998. New insights into cyclosporine A nephrotoxicity by proteome analysis. Electrophoresis 19, 1998–2003.

Anderson, N.L., Esquer-Blasco, R., Hofmann, J.P., Anderson, N.G., 1991. A two-dimensional gel database of rat liver proteins useful in gene regulation and drug effects studies. Electrophoresis 12, 907–930.

Anderson, N.L., Esquer-Blasco, R., Hofmann, J.P., Meheus, L., Raymackers, J., Steiner, S., Witzmann, F., Anderson, N.G., 1995. An updated two-dimensional gel database of rat liver proteins useful in gene regulation and drug effect studies. Electrophoresis 16, 1977–1981.

Anderson, N.L., Taylor, J., Hofmann, J.P., Esquer-Blasco, R., Swift, S., Anderson, N.G., 1996a. Simultaneous measurement of hundreds of liver proteins: application in assessment of liver function. Toxicol. Pathol. 24, 72–76.

Anderson, N.L., Esquer-Blasco, R., Richardson, F., Foxworthy, P., Eacho, P., 1996b. The effects of peroxisome proliferators on protein abundances in mouse liver. Toxicol. Appl. Pharmacol. 137, 75–89.

Arce, A., Aicher, L., Wahl, D., Anderson, N.L., Meheus, L., Raymackers, J., Cordier, A., Steiner, S., 1998. Changes in the liver protein pattern of female Wistar rats treated with the hypoglycemic agent SDZ PGU 693. Life Sci. 63, 2243–2250.

Ayte, J., Gil-Gomez, G., Haro, D., Marrero, P.F., Hegardt, F.G., 1990. Rat mitochondrial and cytosolic 3-hydroxy-3-methylglutaryl-CoA synthases are encoded by two different genes. Proc. Natl. Acad. Sci. USA 87, 3874–3878.

Casals, N., Roca, N., Guerrero, M., Gil-Gomez, G., Ayte, J., Ciudad, C.J., Hegardt, F.G., 1992. Regulation of the expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene. Its role in the control of ketogenesis. Biochem. J. 283, 261–264.

Cunningham, M.L., Pippin, L.L., Anderson, N.L., Wenk, M.L., 1995. The hepatocarcinogen methapyrilene but not the analog pyrilamine induces sustained hepatocellular replication and protein alterations in F344 rats in a 13-week feed study. Toxicol. Appl. Pharmacol. 131, 216–223.

el-Maghrabi, M.R., Lange, A.J., Jiang, W., Yamagata, K., Stoffel, M., Takeda, J., Fernald, A.A., Le Beau, M.M., Bell, G.I., Baker, L., Pilkis, S.J., 1995. Human fructose-1,6-bisphosphatase gene (FBP1): exon–intron organiza-

tion, localization to chromosome bands 9q22.2–q22.3, and mutation screening in subjects with fructose-1,6-bisphosphatase deficiency. Genomics 27, 520–525.

Fujita, T., Inoue, H., Kitamura, T., Sato, N., Shimosawa, T., Maruyama, N., 1998. Senescence marker protein-30 (SMP30) rescues cell death by enhancing plasma membrane Ca^{2+}-pumping activity in Hep G2 cells. Biochem. Biophys. Res. Commun. 250, 374–380.

Fujita, T., Shirasawa, T., Maruyama, N., 1999. Expression and structure of senescence marker protein-30 (SMP30) and its biological significance. Mech. Ageing Dev. 107, 271–280.

Gatlin, C.L., Kleemann, G.R., Hays, L.G., Link, A.J., Yates, J.R., 3rd, 1998. Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. Anal. Biochem. 263, 93–101.

Goldstein, J.L., Brown, M.S., 1984. Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol. J. Lipid Res. 25, 1450–1461.

Goldstein, J.L., Brown, M.S., 1990. Regulation of the mevalonate pathway. Nature 343, 425–430.

Greenspan, M.D., Yudkovitz, J.B., Lo, C.Y., Chen, J.S., Alberts, A.W., Hunt, V.M., Chang, M.N., Yang, S.S., Thompson, K.L., Chiang, Y.C., Chabala, J.C., Monaghan, R.L., Schwartz, R.L., 1987. Inhibition of hydroxymethylglutaryl-coenzyme A synthase by L-659,699. Proc. Natl. Acad. Sci. USA 84, 7488–7492.

Karas, M., Hillenkamp, F., 1988. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal. Chem. 60, 2299–2301.

Kowaluk, E.A., Bhagwat, S.S., Jarvis, M.F., 1998. Adenosine kinase inhibitors. Curr. Pharm. Des. 4, 403–416.

Myers, T.G., Dietz, E.C., Anderson, N.L., Khairallah, E.A., Cohen, S.D., Nelson, S.D., 1995. A comparative study of mouse liver proteins arylated by reactive metabolites of acetaminophen and its nonhepatotoxic regioisomer, 3′-hydroxyacetanilide. Chem. Res. Toxicol. 8, 403–413.

Physicians' Desk Reference, 2000. 54th edn., Medical Economics, Montvale, NJ, p. 2023.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858.

Sirtori, C.R., 1990. Pharmacology and mechanism of action of the new HMG-CoA reductase inhibitors. Pharmacol. Res. 22, 555–563.

Steiner, S., Wahl, D., Mangold, B.L., Robison, R., Raymackers, J., Meheus, L., Anderson, N.L., Cordier, A., 1996. Induction of the adipose differentiation-related protein in liver of etomoxir-treated rats. Biochem. Biophys. Res. Commun. 218, 777–782.

Steiner, S., Gatlin, C.L., Lennon, J.J., McGrath, A.M., Aponte, A.M., Makusky, A.J., Rohrs, M.C., Anderson, N.L., 2000. Proteomics to display lovastatin-induced protein and pathway regulation in rat liver. Electrophoresis 21, 2129–2137.

Swislocki, A.L., Lin, K., Cogburn, D., Fann, K.Y., Khuu, D.T., Noth, R.H., 1997. Postmarketing analysis of lovastatin use in the VA Northern California System of Clinics: a retrospective, computer-based study. Am. J. Manage. Care 3, 1537–1545.