Short Communication

The Effects of Rosuvastatin and the CYP51A1 Inhibitor LEK-935 on the Proteome of Primary Human Hepatocytes

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ABSTRACT:
Elevated amounts of cholesterol are thought to be involved in several severe diseases. Despite the fact that many studies have been performed and published, the effects of cholesterol-lowering agents used to diminish the plasma cholesterol level is not fully understood yet. In this study, the effects of the HMG-CoA reductase inhibitor rosuvastatin and the new CYP51A1 inhibitor 2-((3,4-dichlorophenethyl)(propyl)amino)-1-(pyridin-3-yl)ethanol (LEK-935) on the proteome of primary human hepatocytes were analyzed for the first time. To get an idea about interindividual differences, two different human donors were used. The cytosolic and microsomal fractions of the cells were analyzed by two-dimensional-polyacrylamide gel electrophoresis and capillary high-performance liquid chromatography-mass spectrometry, respectively. Thereby, a set of 44 proteins was found to be differentially presented. The chosen experimental set-up was validated by proteins already known to be affected by statins and involved in the cholesterol biosynthesis. Other proteins found to be regulated cannot be directly related to cholesterol metabolism and have not been described to be affected by cholesterol-lowering agents so far. Some of these proteins may represent interesting targets for further investigations into the analysis of severe side-effects as well as pleiotropic effects of the statins. During the proteome analysis of the two different donors, interindividual differences were observed that were validated by real-time reverse transcription-polymerase chain reaction measurements. Thus, new information and a deeper insight into the processes taking place inside cells treated with cholesterol-lowering agents can be drawn from this study.

Introduction

Elevated ratios of normal cholesterol to high-density lipoprotein cholesterol levels are known to be one of the risk factors of coronary heart disease (Castelli, 1984). These elevated amounts of plasma cholesterol are usually treated by the administration of HMG-CoA reductase inhibitors, blocking de novo cholesterol biosynthesis. For these drugs, severe side-effects (WHO Drug Information, Safety Information, http://apps.who.int/medicinedocs/pdf/h2990e/h2990e.pdf; Yan et al., 2003) as well as pleiotropic effects (LaRosa, 2001) have been described that are not yet fully understood (Liao, 2002; Liao and Laufs, 2005; Corsini et al., 2007). Among the younger generation of synthetic statins, rosuvastatin (RSV) (Smith et al., 2000) is one of the most promising drugs. It is a potent inhibitor of the HMG-CoA reductase and is not metabolized by the major drug-metabolizing cytochromes P450 (McTaggart et al., 2001). An anti-inflammatory, pleiotropic effect of RSV has already been described previously (Stalker et al., 2001). By blocking the HMG-CoA reductase, statins block de novo cholesterol biosynthesis at an early stage of the biosynthetic pathway. In contrast, 2-((3,4-dichlorophenethyl)(propyl)amino)-1-(pyridin-3-yl)ethanol (LEK-935) blocks more downstream of the pathway. It has been shown to be a potent inhibitor of the lanosterol 14α-demethylase (CYP51A1) (Korosec et al., 2008). A detailed comparison of the action of these two drugs, LEK-935 and RSV, has been published recently (Monostory et al., 2009). The present study was aimed to shed light on the biochemical pathways of primary human hepatocytes that are treated with the two cholesterol-lowering agents described above. The results should give deeper insights into the proteins or pathways that may be related to either the pleiotropic effects of the statins still unexplained today or their side-effects. Therefore, our understanding of statin action should be improved.

Human hepatocytes were chosen as a cell model for the present study, because the liver has a high impact on total cholesterol synthesis, uptake of exogenous cholesterol, cholesterol distribution throughout the body, and the maintenance of plasma-cholesterol levels. The liver displays large interindividual differences in its metabolic fluxes, so the use of hepatocytes gained from different donors was thought to be superior to the use of hepatocytes of one
donor or a pool of hepatocytes from different donors in cell culture. By the use of primary cells, the experimental conditions were as near to an in vivo situation as possible. The cytosolic and the microsomal subproteomes contain many proteins involved in cholesterol biosynthesis as well as degradation. Therefore, these two fractions were chosen for analysis. Because the cytosolic fraction contains mainly soluble proteins, it was analyzed by two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE). The digest of the microsomal protein fraction was analyzed by capillary high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS) due to its high content of membrane proteins. In both cases, the analysis was performed in a semiquantitative manner. The proteins were identified by mass spectrometry and ranked into the cellular pathways. The regulation of a chosen set of proteins was validated by real-time, reverse transcription-polymerase chain reaction (RT-PCR) measurements. The results obtained were critically discussed with respect to the experimental design and the potential effects on cellular pathways.

Materials and Methods

Cell Culture. Human livers were obtained from kidney transplant donors or from lobectomy segments resected from adult patients for medical reasons unrelated to our research program (Transplantation and Surgical Clinic, Semmelweis University Budapest, Hungary; Centre Hospitalier Universitaire, Saint-Eloi, Montpellier, France). HH-114 donor was a 57-year-old white woman who died from a ruptured aneurism. HH-129 donor was a 49-year-old white man who died from a stroke. No medication was used before the death of the donors. HH-269 donor was a 46-year-old woman, HH-270 donor was a 57-year-old man, and HH-271 donor was a 56-year-old woman. These three donors had a medical history related to metastasis of colon cancer (HH-270, HH-271) or adenoma on normal cancer (HH-269). Isolation and culture of primary human hepatocytes were performed as described previously (Monostory et al., 2009). Forty-eight hours after serum deprivation, cells were cultured in the presence or absence of inducers (10 μM or LEK-935) for 48 h. Medium was removed and the cells were harvested by scraping.

Sample Preparation. Cell lysis was performed by sonication (USD 30; Emich, Berlin, Germany). The lysate was applied to differential centrifugation. The supernatant, after centrifugation at 170,000g, was defined as the cytosolic fraction and analyzed by 2D-PAGE. The corresponding pellet of the RSV-treated cells was further washed as described previously (Wörner et al., 2009) and analyzed by capillary HPLC-MS. For iTRAQ labeling, the samples were treated according to manufacturer’s instructions (iTRAQ reagents reference guide; Applied Biosystems, Framingham, MA). Protein quantification of samples was done using 2D Quant Kit (GE Healthcare, Munich, Germany).

2D-PAGE. 2D-PAGE was performed with slight modifications as described previously (Böhmer et al., 2006; Hwang et al., 2006). Thereby, an amount of 100 μg of protein was applied per gel. The gels were stained by colloidal Coomassie Blue staining (for a detailed description, see Supplement 1). Image digitalization was done using the Image Master Labscan (GE Healthcare). Quantitative analysis was performed by PDQuest, version 8.0.1, build 055 (Bio-Rad Laboratories, Munich, Germany). Only those spots found to be differentially presented in two different experiments per sample were regarded as true positive hits. For in-gel digestion, gel pieces, approximately 1 x 1 mm in size, were excised from the gels and stored at -20°C, followed by enzymatic (tryptic) digestion (see Supplement 1). Mass spectrometry was performed on a MALDI-TOF/TOF mass spectrometer (ProteomicsAnalyzer 4800; Applied Biosystems). The GPS explorer (Applied Biosystems, Bremen, Germany), equipped with a MASCOT server (version 2.1.03; Matrix Science, London, United Kingdom), was used for protein identification.

HPLC. Off-line, two-dimensional separation of tryptic peptides before mass spectrometric analysis was performed as described previously (Delmotte et al., 2007; Wörner et al., 2009). Of samples 1 and 2, 280 and 90 μg, respectively, were applied for analysis. Mass spectrometry after capillary HPLC-MS was performed as described previously (Wörner et al., 2009). The MASCOT search engine, based on the MOWSE scoring algorithm (Perkins et al., 1999), was used for protein identification. Three different software packages were used for quantitation: ProteinPilot (Applied Biosystems, Framingham, MA); Mascot, and Quant (Boehrnsen et al., 2007). The data were analyzed by the three software packages independently of each other. Only those proteins were regarded as differentially present after RSV treatment that were found by two of the three software packages used. Data export for quantitation by Mascot and Quant was performed using the TS2MASCOT tool (www.matrixscience.com).

Real-Time PCR. The cells used for real-time RT-PCR experiments were treated with rosuvastatin (10 μM) or LEK-935 (10 μM) for 48 h. Total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using a SYBR Green mix, as described previously (Pascussi et al., 2007), and primers sequences are listed in Supplement 1. Cycle thresholds were corrected according to glyceraldehyde-3-phosphate dehydrogenase cycle thresholds and expressed as fold induction compared with control for each donor.

Databases. Information about the proteomes found to be differentially presented was collected using the Expasy/Uniprot database (August 2008) (www.expasy.org), the Kyoto Encyclopedia of Genes and Genomes database (September 2008) (www.genomes.jp/kegg), and by searching the literature. For a detailed description of the procedures described under Materials and Methods, see Supplement 1.

Results and Discussion

General Changes in the Proteome after RSV and LEK-935 Treatment. A set of 44 proteins was found to be regulated after treatment with the cholesterol-lowering agents, rosuvastatin or LEK-935. The detected proteins and their regulations are summarized in Table 1. A detailed description of the proteins found to be regulated is given in Supplement 2.

The Effects of Rosuvastatin on the Proteome of Primary Human Hepatocytes. At first glance, there was almost no correlation between the proteins found to be altered after RSV treatment in the hepatocytes of HH-129 and HH-114 donors. The regulation of only HMG-CoA synthase 1 was similar in all donors (see also Validation of Differential Protein Expression by RT-PCR). The up-regulation of HMG-CoA synthase 1 is obviously a compensatory mechanism of the hepatocytes to overcome the block of the mevalonate pathway and has already been described for other statins (Steiner et al., 2000, 2001). In hepatocytes of both donors, an up-regulation of the cholesterol biosynthesis pathway (in HH-129 cells HMCS1, FDFT1, CPS1, ALCY; in HH-114 cells HMCS1, IDHC) accompanied by an enhanced β-oxidation of fatty acids (in HH-129 cells ACSL; in HH-114 cells D3D2) and by changes in the cholesterol and lipid transport processes (in HH-129 cells APOC1, APOC3; in HH-114 cells ATPB, SAR1B, GRP78) has been detected. This process indicates that the cellular metabolism is driven toward a compensation of the blocked cholesterol biosynthesis. In addition, an increased expression of GSTO1 and PRDX6, protective enzymes against oxidative stress, as well as an enhanced expression of DDAH1, a protein involved in nitric oxide signaling and the maintenance of endothelial function, was found in HH-114 cells. Together with the regulation of those proteins affecting energy metabolism, inflammation, reactive oxygen species protection, and the cytoskeleton, this may help to explain the pleiotropic effects of statins that have been described previously (LaRosa, 2001; Liao, 2002; Liao and Laufs, 2005; Corsini et al., 2007).

The Effects of LEK-935 on the Proteome of Primary Human Hepatocytes. In contrast to RSV treatment, which was found to regulate a bulk of proteins involved in cholesterol and fatty acid
metabolism, LEK-935 affected less proteins involved in these pathways (in HH-129 cells ALBU; in HH-114 cells ATPB, D3D2, GRP78, IDHC). Moreover, no protein involved in the mevalonate pathway was found to be affected by LEK-935 treatment. Thus, LEK-935 obviously does not cause a compensatory mechanism by stimulating cholesterol biosynthesis as does RSV. This effect is due to a different target protein of LEK-935, CYP51A1, which is located more downstream in cholesterol biosynthesis. Therefore, intermediate products of the cholesterol biosynthesis are still present and may diminish a compensatory effect.

Similarly to the effect of RSV, LEK-935 affected proteins involved in the general energy metabolism, in oxidative stress response and inflammation, and in the cytoskeleton organization and transport processes. The effect of RSV on cholesterol and fatty acid metabolism of HH-114 cells, but not HH-129 cells, has been superimposed by the changes in proteins involved in reactive oxygen species protection and inflammation. In contrast, LEK-935 affected such protective proteins in HH-129 cells (ANXA5, IDHP) as well as in HH-114 cells (DDAH1, GSTO1, SODC).

Validation of Differential Protein Expression by Real-Time RT-PCR. A set of 12 proteins was subjected to real-time RT-PCR measurements of samples derived from three different human donors to validate the results of the proteomic analysis (see Table 2). For HMG-CoA synthase 1, the measurements were consistent between the

<table>
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donors analyzed. In four cases (APOC3, DIC, FDF1, and DHB12), changes measured on the proteome level correlated well with those observed by measuring the mRNA levels. The magnitudes of changes observed during the RT-PCR differed between the donors (see Table 2). This finding underlines the huge individual differences between the donors observed by the proteomic analysis. Thus, the RT-PCR measurements underline and validate the results obtained by the proteome analysis.

Summary. The present study describes for the first time the effects of RSV and LEK-935 on the proteome of primary human hepatocytes from two donors. In the cells of both donors, RSV led to an increase in proteins involved in cholesterol biosynthesis, cholesterol transport, and fatty acid β-oxidation. In contrast, LEK-935 showed nearly no compensatory effect on cholesterol biosynthesis. This finding is in accordance with the drug target of LEK-935, CYP51A1, catalyzing one of the last steps of cholesterol biosynthesis. Except for the elevated amounts of HMG-CoA synthase upon RSV treatment, no similarities were observed between the two donors under both treatments, suggesting drastic interindividual variations in the response to the drugs. The underlying reasons for the differences at the single protein level can be manifold, e.g., the dynamic range of the analytical methods or differences in protein expression of the two samples. The activity of a protein is regulated in many ways by levels of various small signal molecules, in addition to the presence or absence of the protein. Nevertheless, the proteins that are identified here to be affected by cholesterol-lowering agents may be used to further investigate the mechanisms of the pleiotropic effects of these drugs.

### Table 2

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<tr>
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<td>1.2</td>
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</tbody>
</table>

APP, appears; DISAP, disappears.


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Sample preparation
Prior to cell lysis, 10 µL of protease inhibitor cocktail (Sigma-Aldrich, Steinheim, Germany) were added to the sample. Cell lysis was performed by sonication (USD 30, Emich, Berlin, Germany). The corresponding parameters were set as following: 4 °C, amplitude 20 µm, 7 x 10 s sonication, 1.5 min break between the sonication steps. The lysate was applied to differential centrifugation (Woerner et al., 2009). The supernatant after centrifugation at 170,000 g was defined as the cytosolic fraction and analysed by 2D gel electrophoresis. The corresponding pellet of the RSV treated cells was further washed as described previously (Woerner et al., 2009) and analysed by capillary LC-MS. Therefore, the final pellet was resuspended in 100 µL dissolution buffer (iTRAQ™ labelling kit, Applied Biosystems). The samples were reduced, alkylated, digested and additionally subjected to iTRAQ™ labelling according to manufacturer’s instructions (Applied Biosystems, iTRAQ™ reagents reference guide). Controls as well as the treated samples were double-labelled. The samples were aliquoted, labelled with the iTRAQ™ labels 114,116 and 115,117 for controls and treated samples, respectively, and finally mixed in a ratio of 1:1:1:1. By this, the experimental procedure was checked for technical variances as the ratios 114/116 and 115/117 could be examined. Protein quantification of samples was done using 2D Quant Kit (GE Healthcare, Munich, Germany).

Two dimensional gel electrophoresis
Two dimensional gel electrophoresis was performed with slight modifications as previously described (Boehmer et al., 2006; Hwang et al., 2006). An amount of 100 µg protein was applied per gel. The sample was loaded by in-gel rehydration over night. The isoelectric focusing (IEF) was performed on the IPGphor, (GE Healthcare, Munich, Germany), using immobilised pH gradient gels (IPGstrips, GE Healthcare, Munich, Germany) with a non-linear pH gradient from 3 – 10 and 18 cm in length. Parameters of isoelectric focussing were as following: the temperature was set to 20 ° C, a maximum current of 50 µA was applied per gel. The focusing protocol was 150 V for 150 Vhrs, 300 V for 600 Vhrs, 600 V for 600 Vhrs, gradient from 600 V to 8,000 V for 4,300 Vhrs, and 8,000 V for 24,000 Vhrs. The following SDS-PAGE was performed as described previously (Boehmer et al., 2006; Hwang et al., 2006).

Staining
The gels were stained by colloidal coomassie blue staining at room temperature by gentle shaking. The volume was set to 0.5 l per gel and the solutions were prepared ready-to-use. After electrophoresis, the gels were applied to fixation (50 % ethanol, 2.55 % phosphoric acid) over night. Thereafter, they were washed two times for 15 minutes prior to incubation in pre-incubation solution (34 % methanol, 17 % ammoniumsulfate, 2.55 % phosphoric acid) for 1.5 h. The Coomassie brilliant blue was directly added to the shaking pre-incubation
solution and the gels were incubated for 5 days. Destaining was performed by 2 – 3 times washing in ultra-pure water.

Quantitative analysis
Image digitalisation was done using the Image Master Labscan, GE Healthcare (Munich, Germany). Quantitative analysis was performed by PDQuest version 8.0.1 build 055 (BioRad, Munich, Germany). Only those spots found to be differentially present in two different experiments per sample were regarded as true positive hits.

In-gel digestion
Unless otherwise stated, all steps of the excision and digestion process were performed at room temperature. For in-gel digestion, gel pieces, approximately 1 x 1 mm in size, were excised from the gels and stored at –20 °C prior to their enzymatic digestion.

First, the gel plugs were washed for 10 minutes in 50 µl water, followed by incubation for 15 minutes in 50 µl of an 50 % acetonitrile (ACN) solution; while they were shaken at 1 200 rpm, in intervals of 1 minute shaking followed by 1 minute break in a Thermomixer comfort (Eppendorf, Hamburg, Germany). Thereafter, the gel plugs were destained by incubation in 50 µl ACN for 1 minute, followed by 5 minutes incubation in 20 µl bicarbonate buffer (40 mM) at 450 rpm. After adding 20 µl ACN, the solution was further incubated for 15 minutes at 450 rpm. The destaining procedure was repeated until the dye was completely vanished. Thereafter, the plugs were dried in a speed vac and digestion was started by adding 15 µl of a trypsin solution (27 ng/µl in bicarbonate buffer (40 mM)). Digestion was performed at 37 °C over night and stopped by freezing the peptide solution in liquid nitrogen.

Prior to mass spectrometric analysis, the peptide solution was concentrated using ZipTips™ (Millipore, Billerica, MA, USA) according to supplier’s instructions. The sample was directly eluted in 5 µl of matrix solution (3 % alpha-cyano-4-hydroxy-cinnamic acid (CHCA) in 50 % ACN, 0.1 % TFA). Of the eluate, 2 x 1 µl was spotted onto a MALDI target (Applied Biosystems, Bremen, Germany).

Mass spectrometry after 2D gel electrophoresis and in-gel digestion
Mass spectrometry was performed on a MALDI-TOF/TOF mass spectrometer (ProteomicsAnalyzer 4800™, Applied Biosystems, Framingham, MA, USA). In MS mode, mass spectra were acquired in a mass range of 800 to 4000 m/z, with the focus mass set to 2100 m/z. Laser intensity was adapted for each measurement separately, per sub-spectrum acquired, 50 laser shots were summarised. In total, eight sub-spectra were accumulated (400 shots). For data processing, the following parameters were set for peak filtering: raw spectrum filtering: subtract baseline; peak width: 50; smooth: no. Peak detection was performed according to the following settings: minimal S/N: 5; local noise window width (m/z): 50; minimal peak width at full width half max (bins): 2.9. The expected mass to resolution relations were: 1200 m/z resolution of 17,000; 2400 m/z resolution of 22,000 and 3600 m/z resolution of 17,000. The
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Monoisotopic peaks were flagged (adduct was set to H) and the cluster area S/N optimisation was enabled with a S/N threshold of 10. The interpretation method for precursor selection contained the following parameters: minimal S/N: 35; adduct exclusion (Da): 21.982 and 37.956; adduct tolerance (m/z): ± 0.03; exclude precursors within: 200. A maximum of five precursors was chosen per spot. In MS/MS mode fragmentation was realised using the CID chamber with air as collision gas. The precursor mass window was set to a resolution of 200,000 and the metastable suppressor was turned on. Here again, laser intensity was manually adjusted prior to each measurement. In total, 40 sub-spectra with 50 shots per sub-spectrum were acquired and accumulated to the final MS/MS spectrum (2000 shots). The data processing settings were the same as for MS measurements, except for the parameters of peak detection. Changes were: the minimal S/N was set to 3, the local noise window width to 250 m/z and the expected mass to resolution relations were: 100 m/z resolution of 4000; 500 m/z resolution of 4500, 1000 m/z resolution of 7000 and 1500 m/z resolution of 6000. To achieve an optimal mass accuracy, the instrument was calibrated in MS and MS/MS mode using a six-peptide calibration mix provided by Applied Biosystems spread over thirteen calibration spots on the MALDI target. The peak matching settings for the internal calibration in MS/MS mode were as follows: minimal S/N: 10; mass tolerance (m/z): ± 1; minimum number of peaks to match: 5; maximal outlier error (ppm): 25; use monoisotopic peaks only: yes. The weighted fit was set to “equal”. Fragments for the glufib1 peptide (1560.677 m/z) of the calibration mix were used for calibration.

Identification of proteins
The MASCOT search engine based on the MOWSE scoring algorithm (Perkins et al., 1999) was used for protein identification. The GPS explorer (Applied Biosystems, Bremen, Germany) equipped with a MASCOT server (version 2.1.03) was used. A combined MS and MS/MS search was performed. The peak filtering parameter for MS data were as follows: mass range, 800 – 4000 Da; minimum S/N, 10; maximum peak density, 20 per 200 Da, maximum number of peaks, 65. The filtering parameter for MS/MS data were the same except for the mass range, which was set to 60 Da to precursor mass minus 35 Da. For identification, the following parameters were applied: taxonomy: no restriction; fixed modifications: none; variable modifications: carbamidomethyl (C), oxidation (M); enzyme: trypsin; maximum missed cleavages: 1; precursor tolerance: 50 ppm, MS/MS tolerance: 0.1 Da; maximum peptide rank: 10. Search was performed against swissprot database (version 56.0). A GPS score of 100 % was mandatory.
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High-performance liquid chromatography (HPLC)
Off-line two-dimensional separation of tryptic peptides before mass spectrometric analysis was performed as described previously (Delmotte et al., 2007; Woerner et al., 2009) using RP x IP-RP HPLC with a basic eluent in the first and an acidic eluent in the second dimension. Of sample 1, 280 µg and of sample 2, 90 µg were applied for analysis.

Mass spectrometry after HPLC
Mass spectrometry after capillary HPLC-MS and spotting onto a MALDI-target was performed as described previously (Woerner et al., 2009).

Identification after capillary HPLC-MS
The MASCOT search engine, based on the MOWSE scoring algorithm (Perkins et al., 1999) was used for protein identification. The following parameters were applied: taxonomy: *homo sapiens*; fixed modification: iTRAQ 4-plex (N-term), iTRAQ 4-plex (K); variable modification: iTRAQ 4-plex (Y), methylthio (C), methionine oxidation; enzyme: trypsin; peptide tolerance: 50 ppm; MS/MS tolerance: ± 0.2 Da. The samples were searched against swissprot database (version 54.7; January 15th, 2008; downloaded from www.matrixscience.com). ProteinPilot, version 2 (Applied Biosystems, Bremen, Germany) was used for quantitation (see below) and identification. The parameters applied for protein identification by the paragon algorithm were as follows: sample type: iTRAQ 4-plex (peptide labelled); Cys alkylation: MMTS; digestion: trypsin; special factors: no; species: *homo sapiens*; ID focus: biological modifications; search effort: thorough ID; detected protein threshold: >2.0 (99 %).

Quantitation
Three different software packages were used for quantitation. ProteinPilot (Applied Biosystems, Framingham, MA, USA) was chosen as quantitation and identification software available from Applied Biosystems, able to interact directly with the mass data stored in the instruments Oracle database. Mascot was chosen as the most prominent identification tool, serving as a quantitation tool, too. Finally Quant, a freely available software package for quantitation was used (Boehm et al., 2007). It uses Mascot result files as a starting point, as it does not contain an algorithm for identification.

Only those proteins were regarded as differentially present after RSV treatment that were found by two out of the three software packages used.

Data export for quantitation by Mascot as well as Quant was performed by the TS2MASCOT – tool (www.matrixscience.com). For Mascot, the quantitation parameters were as follows: Constrain search: no; protein ratio type: weighted; protein score type: mudpit; report detail: yes; show subsets: two; require bold red: yes; minimum peptides: two; significance threshold: 0.05; modification groups: iTRAQ (Y) variable, iTRAQ4plex; protocol: reporter; integration method: none. Quality settings were: minimum precursor charge: 1; isolated precursor: no; minimum a(1): 0.0; peptide threshold: at least identity;
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exclusion: iTRAQ (Y) variable. No outlier removal was performed and normalisation method was set to median.

Using Quant, the following parameters were set: reporter tolerance: ±0.5 Da; intensity range: no settings; absolute intensity error: 0.5 cts; experimental error: 0 %; only unique peptide: yes; Mascot significance threshold: p 0.05. The “mresx_1265.exe” application was used to handle the MASCOT result files.

Real-time PCR

To validate differential protein expression, the expression of some proteins found in altered amounts after the treatment with cholesterol lowering agents was also checked by real-time polymerase chain reaction (RT-PCR). The primers used were designed on spanning exon boundaries using www.primer3. They are listed in Table 1. Total RNA samples from three different primary cultures of human hepatocytes were used. The cells were treated for 48 hours with rosuvastatin (10 µM) or LK-935 (10 µM). cDNAs were synthesized from 500 ng of total RNA using the MMLV (Invitrogen, Cergy Pontoise Cedex, France) at 37 °C for 60 min, in the presence of random hexamers (GE Healthcare, Munich, Germany), and then diluted 10-fold in water. RT-PCR amplifications were performed using a SYBR Green mix and a Mx3000P apparatus from Stratagene (La Jolla, CA, USA). Cts were corrected according to GAPDH Cts and expressed as fold induction compared to control for each donor.

Table 1 Primers used for the RT-PCR experiments, their sequence, melting temperature as well as the product size

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<th>Primer forward</th>
<th>Primer reverse</th>
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Drug metabolism and disposition

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Databases

Information about the proteins found to be differentially present was collected using the Expasy/Uniprot database (August 2008) (www.expasy.org), the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (September 2008) (www.genomes.jp/kegg) and by searching the literature.

References


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Supplement 2 – detailed information on proteins
Discussion of the changes found in the protein levels shown in Table 1 of the manuscript:

The effects of RSV
Cholesterol metabolism
In HH-129 cells, four of the proteins present in higher amounts after RSV treatment are directly related to cholesterol biosynthesis. The ATP citrate lyase (ACLY), found to be up-regulated, plays a crucial role in the synthesis of cytoplasmic acetyl-CoA (Wang et al., 2009), being a substrate of the cytoplasmic HMG-CoA synthase 1. This protein was found to be more than three times up-regulated and it catalyses the synthesis of HMG-CoA. Beside these enzymes delivering precursor molecules for the cholesterol biosynthesis and acting at early steps of the mevalonate pathway, two more enzymes involved in the sterol-targeted branch of the pathway were found to be up-regulated in HH-129 cells treated with RSV. The squalene synthase (FDFT), the key enzyme for the metabolite flow into the sterol branch of the mevalonate pathway (Do et al., 2009) as well as the lanosterol 14-demethylase (CYP51) were detected at elevated amounts.

The expression of these four proteins, HMCS1, FDT1, CYP51 and ACLY, is regulated, at least in part, by the sterol regulated element binding protein 2 (SREBP2). In contrast to SREBP1 that activates de novo fatty acid synthesis, SREBP2 is known to be mainly responsible for the regulation of cholesterogenic enzymes (Horton et al., 2002).

Beside the proteins involved in cholesterol biosynthesis, the apolipoproteins C-I and C-III (APOC1, APOC3) were found in increased amounts. The members of the apolipoprotein C family (ApoC) are protein constituents of chylomicrons, VLDL and HDL particles (Jong et al., 1999) and thereby involved in cholesterol transport processes in the human body.

In HH-114 cells treated with RSV, two proteins related to cholesterol biosynthesis were found in increased amounts. The cytoplasmic isocitrate dehydrogenase (IDHC) plays a key role in lipogenesis by supplying the NADPH for fatty acid and cholesterol biosynthesis (Shechter et al., 2003; Koh et al., 2004). In addition, the HMG-CoA synthase 1, necessary for the production of HMG from acetyl-CoA and acetoacetyl-CoA, was found in increased amounts. Thus, enzymes involved into the biosynthesis of cholesterol and acting at key positions of this pathway were found to be up-regulated in cells derived from both donors.

Proteins involved in cholesterol transport were also found to be changed in HH-114 cells after RSV treatment. The beta-chain of mitochondrial ATP synthase (ATPB) was found to be up-regulated. This protein has recently been shown to be a high affinity receptor of apolipoprotein A1 and apolipoprotein E (Martinez et al., 2003). It thereby triggers the endocytosis of HDL particles. The second protein of HH-114 cells involved in cholesterol transport is the GTP-binding protein SAR1b, required for the intracellular
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transport of cholesterol by chylomicrons and VLDL (Shoulders et al., 2004). Mutations of this protein are the reason for Anderson’s disease that has recently been shown to be related to myolysis (Silvain et al., 2008). Finally, the 78 kDa glucose-regulated protein which was found in reduced amounts is also involved in cholesterol transport processes. This protein catalyses the rate-limiting step in LDL maturation (Jorgensen et al., 2000). Taken together, analysis of the hepatocytes from two donors clearly demonstrates that RSV on the one hand, inhibits cholesterol production, but, on the other hand, also causes compensatory pathways aimed to improve cholesterol biosynthesis and transport.

Fatty acids
Among the regulated proteins of HH-129 cells, two, the long-chain fatty acid CoA ligase (ACSL) and the estradiol 17-beta-dehydrogenase 12 (DHB12) are involved in fatty acid synthesis and degradation. Both were found in elevated amounts after RSV treatment. The ACSL produces long-chain fatty acid acyl CoAs that have an inhibitory effect on the acetyl-CoA carboxylase, catalysing the initiation step of de novo fatty acid synthesis (Hardie, 1989). The beta-oxidation of fatty acids is started by the ACSL (Schoonjans et al., 1995). The up-regulation of this pathway has already been described for another statin, lovastatin (Singh et al., 1998). The 17-beta dehydrogenases catalyse the transformation of estrone to estradiol and is involved in fatty acid elongation, acting as a reductase of long-chain fatty acid acyl-CoA as well as 3-keto-acyl-CoA (Moon and Horton, 2003). Interestingly, only a poor correlation of the enzyme to estradiol levels is reported (Nagasaki et al., 2009).

In HH-114 cells, also an activated beta-oxidation of fatty acids has been suggested due to the increased amounts of mitochondrial 3,2 trans-enoyl-CoA isomerase (D3D2). This enzyme catalyses a key-step of beta-oxidation of unsaturated fatty acids in two compartments peroxisomes (Hiltunen et al., 1996) and mitochondria (Janssen et al., 1994; Stoffel et al., 1994). Thus, in both samples, proteins involved in beta-oxidation were found to be up-regulated upon RSV treatment.

Energy metabolism
In HH-129 cells, the energy metabolism may be affected by the increased amount of C560 as well as the decreased amount of phosphoglucomutase 1 (PGM1) that catalyses the conversion of glucose-1-phosphate to glucose-6-phosphate. This step is necessary to use the glucose-1-phosphate, gained during glycogen decomposition, for glycolysis. The mannose-P-dolichol utilisation defect protein 1, found in increased amounts, is a key protein of glycosylation catalysing the first step of this pathway (Anand et al., 2001). In HH-114 cells, four of the differentially present proteins were found to be involved in energy metabolism. The voltage-dependent anion-selective channel 2 (VDAC2) found in increased amounts mediates the flow of ATP through the mitochondrial membrane and thereby plays a crucial role in energy metabolism (Rostovtseva and Colombini, 1997). The increased
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amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) points towards an increase in glycolysis. Another part of the energy metabolism was affected by the increased amounts of ketohexokinase involved in fructose metabolism (Heinz et al., 1968). A second enzyme of the fructose metabolism, the fructose-bisphosphate aldolase B (ALDOB), responsible for the metabolism of fructose 1-phosphate (Lebherz and Rutter, 1969) was found in decreased amounts in HH-114 cells. Thus, although RSV seems to affect the energy metabolism of the cells, this effect seems to be rather complex.

Inflammation, Apoptosis, Oxidative stress
In HH-114 cells, the effects on cholesterol and fatty acid metabolism are superposed by increased amounts of proteins involved in the protection against oxidative stress and inflammation. Two of the proteins found in increased amounts are directly related to the protection against oxidative stress. The glutathione-S-transferase omega 1 (GSTO1) is not involved in xenobiotic detoxification as other glutathione transferases, but it shows a functionality comparable to glutaredoxins and may play a critical role in the protection against oxidative stress by restoring enzymatic activity formerly blocked by S-thiol formation (Board et al., 2000). Furthermore, it was shown to be involved in cytokine signalling and apoptosis (Laliberte et al., 2003). The second enzyme is peroxiredoxin 6 (PRDX6), which is reduced by glutathione and shows a protective effect against oxidative stress (Manevich et al., 2009). Moreover, the voltage-dependent anion-selective channel protein 2 (VDAC2_HUMAN) was found in increased amounts. It controls the release of superoxide anions from mitochondria to the cytosol (Han et al., 2003). VDAC proteins affect the activity of the copper-zinc superoxide dismutase (SODC) (Budzinska et al., 2007) a protein found in decreased amounts in the cytosol of HH-114 cells. The N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 (DDAH1) was found in increased amounts after RSV treatment. It increases basal levels of vascular NO and thereby protects against endothelial dysfunction induced by asymmetric dimethylarginine (Dayoub et al., 2008). The fibrinogen alpha and beta, found to be up-regulated, belong to the group of acute phase proteins being involved in inflammation (Redman and Xia, 2000). For GAPDH, which is up-regulated, different biological activities in apoptosis and proliferation of hepatocytes are described (Sirover, 1999) (Barbini et al., 2007), besides its glycolytic activity. Among others, it is involved in tubulin bundling (Sirover, 1999), interacts with glutathione (Sirover, 1999) and shows a hepatoprotective effect against oxidative stress (Kuo and Slivka, 1994).

Others
Two of the proteins found in increased amounts in HH-114 cells are related to the cytoskeleton. The first one is tubulin, which is part of the microtubules (Desai and Mitchison, 1997). The second one is radixin as part of the anchor structures that
Supplement 2 – detailed information on proteins

connect the cytoskeleton to the plasma membrane (Sato et al., 1992). The mitochondrial dicarboxylate carrier (DIC) was found in increased amounts. Lin et al. (2005) described an increased ROS production and hyperpolarisation of the mitochondrial membrane after DIC overexpression, whereas Zhong et al. (2008) described a ROS protective effect due to the involvement of DIC in glutathione transport into the rat liver mitochondria. The protein is also involved in glyceroneogenesis (Reshef et al., 2003). Its mRNA is induced by fatty acids (Reshef et al., 2003), while its activity is inhibited by long-chain fatty acid acyl CoAs (Ventura et al., 2005) and the enzyme itself mediates the protonotrophic action of long chain fatty acids (Wieckowski and Wojtczak, 1997). Moreover, the over-expression of DIC also led to an increased up-take of succinate, which is fed into complex II, a step of the respiratory chain (Lin et al., 2005). The carbonic anhydrase 2 (CAH2), found in increased amounts, catalyses the formation of bicarbonate from carbon dioxide. The eukaryotic initiation factor 4A found to be down-regulated in HH-114 cells exhibits RNA helicase activity. Finally, the retinal dehydrogenase 1 (AL1A1) being down-regulated by RSV metabolises 4-hydroxynonenal (Esterbauer et al., 1985) a product formed during lipid peroxidation.

The effects of LEK-935

Similarly to RSV treatment, exposure to LEK-935 also led to changes in the protein pattern. Proteins involved in the cholesterol metabolism as well as energy metabolism and proteins related to inflammation, apoptosis and oxidative stress were found to be regulated by LEK-935 treatment.

Cholesterol metabolism

No protein directly involved in the cholesterol biosynthetic pathway has been found in altered amounts after LEK-935 treatment of HH-129 cells. However, one of the proteins down-regulated, serum-albumin, is related to cholesterol transport. It is the major protein in the plasma, and also binds fatty acids (Spector, 1975) as well as steroid hormones (Pardridge and Mietus, 1979), thereby serves as a transporter of these molecules. In HH-114 cells, cholesterol metabolism was affected by the regulation of the IDHC, delivering the NADPH necessary for fatty acid and cholesterol biosynthesis. The ATPB, found in increased amounts and the GRP78 found in decreased amounts can be related to cholesterol transport as described for RSV treatment of HH-114 cells.

Fatty acids

None of the proteins found to be regulated by LEK-935 in HH-129 cells can be related to fatty acids. In HH-114 cells, the increased amounts of D3D2, similarly to RSV treatment, suggests an activated beta oxidation of fatty acids.

Energy metabolism

The only protein involved in energy metabolism found in altered amounts in HH-129 cells treated with LEK-935 is the GAPDH. It has also been found in elevated amounts after RSV treatment in HH-114 cells. After LEK-935 treatment, two proteins related to energy metabolism were changed in HH-114 cells. The ketohexokinase
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involved in fructose metabolism (Heinz et al., 1968) was found in increased amounts. A second enzyme of the fructose metabolism, the ALDOB was found in decreased amounts in HH-114 cells treated with not only RSV, but also with LEK-935.

Inflammation, Apoptosis, Oxidative stress

Two of the proteins found in altered amounts in HH-129 cells are related to protection against ROS or inflammation. Annexin A5 (ANXA5) found in elevated amounts shows anticoagulant (Thiagarajan and Tait, 1990) and anti-inflammatory effects (Reutelingsperger and vanHeerde, 1997). We demonstrated elevated amounts of mitochondrial isocitrate dehydrogenase [NADP] (IDHP) which is the major NADPH producer in mitochondria, whose up-regulation has been shown to enhance the protection against ROS (Jo et al., 2001). In addition, NADPH is also necessary for fatty acid and cholesterol biosynthesis. In HH-114 cells, three of the proteins that changed after RSV treatment are involved in the protection against oxidative stress and inflammation and were also found to be regulated by LEK-935 treatment. The GSTO1 that may play a critical role in the protection against oxidative stress as well as DDAH1 possibly protecting against endothelial dysfunction were found in increased amounts while the SODC was found in decreased amounts.

Others

Both actin and radixin which are involved in the cytoskeleton function (Bretscher, 1991; Sato et al., 1992) were found in reduced amounts after LEK-935 treatment of HH-129 cells. The nicotinamide N-methyltransferase (NNMT) was also found in reduced amounts. A reduced activity of the NNMT could cause a decrease in nicotinamide excretion. This would lead to increased NAD or NADP synthesis, which is necessary for ATP and cholesterol biosynthesis, respectively. As a second product of the reaction catalysed by NNMT, homocysteine is formed. Souto et al. (2005) suggested that NNMT is the major source of plasma homocysteine. Elevated plasma levels of homocysteine are associated with an increased risk of cardiovascular diseases. For simvastatin, a decrease in plasma homocysteine has already been described (Luftjohann et al., 2001).

In HH-114 cells, two proteins involved in the cytoskeleton structure were found in increased amounts. Tubulin, which is part of the microtubules (Desai and Mitchison, 1997) and actin as a part of the actin fibres of the cytoskeleton. Similarly to RSV effect, the CAH2 involved in the buffering system of the cell was found in increased, whereas the AL1A1 involved in lipid peroxidation in decreased amounts.

It has to be mentioned, that except for the cytoplasmic actin, all of the proteins found to be regulated by LEK-935 in HH-114 cells had also been regulated by RSV treatment.
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