Proteomic Profiling of Hepatic Endoplasmic Reticulum-associated Proteins in an Animal Model of Insulin Resistance and Metabolic Dyslipidemia*

Jean-Paul F. Morand‡§, Joseph Macri¶, and Khosrow Adeli||

From the ‡Division of Clinical Biochemistry, and Structural Biology and Biochemistry, Department of Pediatric Laboratory Medicine, Research Institute, The Hospital for Sick Children and the University of Toronto, Toronto, Ontario M5G 1X8 and the ¶Department of Laboratory Medicine, Hamilton Regional Laboratory Medicine Program and McMaster University, Hamilton, Ontario L8L 2X2, Canada

Hepatic insulin resistance and lipoprotein overproduction are common features of the metabolic syndrome and insulin-resistant states. A fructose-fed, insulin-resistant hamster model was recently developed to investigate mechanisms linking the development of hepatic insulin resistance and overproduction of atherogenic lipoproteins. Here we report a systematic analysis of protein expression profiles in the endoplasmic reticulum (ER) fractions isolated from livers of fructose-fed hamsters with the intention of identifying new candidates involved in hepatic complications of insulin resistance and lipoprotein dysregulation. We have profiled hepatic ER-associated proteins from chow-fed (control) and fructose-fed (insulin-resistant) hamsters using two-dimensional gel electrophoresis and mass spectrometry. A total of 26 large scale two-dimensional gels of hepatic ER were used to identify 34 differentially expressed hepatic ER protein spots observed to be at least 2-fold differentially expressed with fructose feeding and the onset of insulin resistance. Differentially expressed proteins were identified by matrix-assisted laser desorption ionization-quadrupole time of flight (MALDI-Q-TOF), MALDI-TOF-postsource decay, and database mining using ProteinProspector MS-fit and MS-tag or the PROWL ProFound search engine using a focused rodent or mammalian search. Hepatic ER proteins ER60, ERP46, ERP29, glutamate dehydrogenase, and TAP1 were shown to be more than 2-fold down-regulated, whereas α-glucosidase, P-glycoprotein, fibrinogen, protein disulfide isomerase, GRP94, and apolipoprotein E were all found to be up-regulated in the hepatic ER of the fructose-fed hamster. Seven isoforms of ER60 in the hepatic ER were all shown to be down-regulated at least 2-fold in hepatocytes from fructose-fed/insulin-resistant hamsters. Implications of the differential expression of positively identified protein factors in the development of hepatic insulin resistance and lipoprotein abnormalities are discussed.

Proteomics involves the integration of a number of technologies with the aim of analyzing the complete complement of proteins (referred to as the proteome) expressed by a biological system in response to various stimuli and/or under different physiological or pathological conditions. Examining changes in the proteome offers insight into cellular and molecular mechanisms that cannot always be obtained through genomic analysis. The information gap between the genome and cellular processes resulting from gene products can be largely attributed to post-translational modifications such as phosphorylation and glycosylation. These modifications, which cannot be monitored using only genomic analyses, have been shown to modulate important regulatory processes such as protein turnover, protein activity, and protein localization within a cell. In addition, virtually all known cellular signaling pathways are mediated through a complex cascade of reversible protein phosphorylation. The proteome is a dynamic feature, subject to changes resulting from developmental stage, disease state, or environmental conditions. An important goal of proteomics-based research is to incorporate the information obtained from these types of analysis into a comprehensive proteome database containing both protein identification and their corresponding functional characteristics.

Metabolic dyslipidemia and cardiovascular disease are regarded as major complications of the metabolic syndrome (1). Key mechanistic features of metabolic dyslipidemia include increased hepatic secretion of very low density lipoprotein (VLDL),1 decreased antiatherogenic high density lipoprotein (HDL) cholesterol, elevated atherogenic small dense LDL particles (2), and increased enteric secretion of apoB48-containing chylomicrons (3). A diet-induced animal model of insulin resistance, the fructose-fed Syrian golden hamster, was recently employed to investigate cellular and molecular mechanisms involved in the development of metabolic dyslipidemia. Fructose feeding for a 2-week period induced whole body insulin resistance (4). Induction of insulin resistance was accompanied by a considerable rise in hepatic VLDL-apoB and triglyceride production (4). Evidence was also obtained indicating impaired hepatic insulin signaling and insulin resistance in the liver of fructose-fed hamsters (5). Interestingly, amelioration of insulin resistance in these hamsters was associated with improved hepatic glucose metabolism (6). The mechanisms linking the development of hepatic insulin resistance and lipoprotein overproduction need to be better understood.

1 The abbreviations used are: VLDL, very low density lipoprotein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ER, endoplasmic reticulum; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; MALDI, matrix-assisted laser desorption ionization; MHC, major histocompatibility complex; LDL, protein disulfide isomerase; PSD, postsource decay; Q-TOF, quadrupole time of flight; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; TOF, time of flight; α-LAMP-1, lysosomal-associated membrane protein-1.
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resistance in these hamsters using the insulin-sensitizing agent rosiglitazone significantly reduced hepatic lipoprotein production, suggesting a direct link between insulin sensitivity and lipoprotein abnormalities (6).

We have identified a number of key molecules, including protein-tyrosine phosphatase 1B, in the livers of fructose-fed hamsters which appear to link hepatic insulin signaling with alterations in the synthesis and secretion of lipids and lipoproteins (5, 7). These studies would undoubtedly be enhanced and complemented by employing a proteomics approach to analyze changes in the protein profile of key insulin-sensitive tissues in various dietary models of insulin resistance. A proteomics-based approach has the best potential for identifying a large number of changes in proteins accompanying the development of insulin resistance and metabolic dyslipidemia. Future research using animal models of insulin resistance would greatly benefit from the establishment of a two-dimensional protein database derived from insulin-sensitive tissues, including liver, muscle, adipose, and small intestine, in the fructose-fed hamster model. Such a database would provide information that may greatly facilitate our understanding of the mechanisms underlying insulin resistance and the accompanying dyslipidemia.

Because a number of key signaling events as well as the pathways responsible for lipoprotein assembly occur in the endoplasmic reticulum (ER) compartment of hepatocytes, the current study focused on the identification of differentially expressed proteins in the ER fraction of hepatocytes derived from chow-fed and fructose-fed hamsters. Here we report the complete profile of ER-associated proteins from insulin-resistant and control hepatocytes by two-dimensional gel electrophoresis as well as the identification of a number of differentially expressed protein factors that may be important in the development of hepatic insulin resistance and the associated lipoprotein abnormalities.

EXPERIMENTAL PROCEDURES

Animal Surgery and Liver Perfusion—Surgery and liver perfusion were performed essentially as described previously (4, 8). Briefly, male Syrian golden hamsters (Charles River, Montreal, Canada) were fed either a normal chow diet or a 60% fructose diet (Dyets, Inc, Bethlehem, PA) for 2 weeks and fasted overnight prior to sacrifice. Surgery was conducted on a heating pad to maintain body temperature. The liver was perfused by isolating it from the circulatory system; the thoracic aorta, left renal, portal, and vena cava, the abdominal aorta, and the portal, hepatic, and vena cava were blocked using sutures. The portal vein was then severed, allowing the flow of 50 ml of 37 °C liver perfusion medium out of the liver (In vitrogen). After this, 50 ml of 37 °C liver digest medium containing collagenase (In vitrogen) was circulated through the liver.

The liver was excised and minced in hepatocyte wash medium (Invitrogen).

Subcellular Fractionation and Isolation of Hepatic Endoplasmic Reticulum—After perfusion the liver was passed through a 100 μm nylon cell strainer (BD Falcon, Bedford, MA), allowing for the isolation of individual hepatocytes. Three washes using ice-cold phosphate-buffered saline with the hepatocytes being pelleted at 500 rpm at 4 °C were performed to remove collagenase. Hepatocytes were resuspended in lysis buffer (0.25 μM sucrose, 10 mM Tris-HCl, pH 7.4) containing a protease inhibitor mixture (Complete-Mini, EDTA-free, Roche Molecular Biochemicals) and homogenized with 10 strokes of a Teflon and glass Dounce homogenizer (Wheaton, Millville, NJ). The homogenate was centrifuged at 1,000 × g for 10 min at 4 °C to pellet unhomogenized cells, plasma membrane, and nuclei. The supernatant was centrifuged at 15,000 × g for 16 min in an Optima LE-80 ultracentrifuge using a SW50.1 rotor (Beckman-Coulter) at 4 °C to pellet the mitochondria, lysosomes, peroxisomes, and Golgi. The supernatant was again subjected to ultracentrifugation using a SW55-Ti rotor at 100,000 × g for 50 min at 4 °C. The pellet was resuspended in 54% Iodixanol (Optiprep, Axis Shield/Cedarlane, Hornby, ON, Canada) and subjected to ultracentrifugation at 350,000 × g for 2.12 h at 4 °C using a V65.2-Ti vertical rotor. After this spin, 20 fractions were unloaded from the top of the tube in 245-μl volumes, and a protein assay was performed on each fraction. The subcellular fractions were prepared for immunoblotting by combining 21 μl of each fraction with 9 μl of sample buffer (8% SDS, 0.25 μM Tris-HCl, 40% glycerol, 1% β-mercaptoethanol) and boiled for 5 min at 100 °C to denature the protein fully. The fraction control, consisting of homogenized whole microsome, kept from an earlier step in the fractionation process, were subjected to electrophoresis on 8% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were immunoblotted with antibodies to either of the ER markers calnexin (Santa Cruz Biotechnology, Santa Cruz, CA) or ER60 (a gift from Dr. Reiko Urade, Kyoto University, Japan). The immunoblots were exposed to film, and the intensity of each protein band was quantified by densitometry. Antibodies also used to test the abundance and purity of ER present in each fraction were α-catalase (peroxisomal marker) (a gift from Dr. Ingrid Tein, Hospital for Sick Children), α-49K (mitochondrial marker) (a gift from Dr. Ingrid Tein), α-58K (Golgi marker) (Sigma), α-LAMP-1 (lysosomal marker) (Santa Cruz Biotechnology), and α-p53 (Santa Cruz Biotechnology). Fractions determined to be free of contaminating organelles by immunoblot analysis using these antibodies were prepared for two-dimensional gel electrophoresis.

Proteomics—The most concentrated/pure hepatic ER fractions were pooled and used for two-dimensional electrophoresis. The samples were diluted with 2 volumes of lysis buffer and subjected to ultracentrifugation using a SW55-Ti rotor (Beckman-Coulter) at 4 °C to pellet the ER microsomes. The protein pellet was resuspended in a commercially available protein-solubilizing solution (Bio-Rad Sequential Protein Extraction Kit: 5 μl urea, 2 μl thiourea, 2% w/v CHAPS, 2% w/v SDS 3–10, 40 mM Tris, 0.2% w/v Bio-Lyte 3–10 ampholyte, and 1% w/v trityl phosphine) and vortexed for 5 min. After the sample preparation, a protein assay was performed on the two-dimensional preparations using the Bio-Rad 50 μl protein assay (Bio-Rad). Next, 200 μg of protein (or up to 2 mg for preparative gels) was made up to 450 μl with rehydration buffer (8 μl urea, 0.5% w/v CHAPS, 15 mM dithiothreitol, 0.2% w/v Pharma Lyte 3–10) for the 24-cm Immobiline Drystrips (Amersham Biosciences). Alternatively, 25 μg of protein was made up to 150 μl with rehydration buffer for the 7-cm Immobiline Drystrips.

The first dimension was performed using IEF/phoretic isoelectric focusing using 7-cm, 10-cm, and 24-cm 3–10 NL Immobiline Drystrips allowed to swell for at least 12 h prior to focusing (Amersham Biosciences). A step-and-hold pattern was used to reach 30 V for 1 h, 150 V for 1 h, 300 V for 1 h, 1,000 V for 1 h, and 2,500 V for 1 h. A gradient pattern was used next to reach 8,000 V for 1 h followed by step-and-hold to 8,000 V for 3 h, and then into a 150-V holding pattern.

After focusing, strips were soaked in equilibration buffer (6 μl urea, 300 μg/ml CHAPS, and 2% w/v SDS in 0.5% Tris- HCl buffer) plus 1% diethiothreitol for 15 min, followed by 15 min in equilibration buffer with 4.8% iodoacetamide. The 24-cm strips were loaded onto in-lab cast 24 × 22-cm 12% SDS-polyacrylamide gels and sealed with 0.5% agarose made up in 1 × running buffer. All gels comprising an experiment were electrophoresed simultaneously in the Ettan DALTsix large format (Bio-Rad) Next, 200 μg of protein (or up to 2 mg for preparative gels) was made up to 450 μl with rehydration buffer (8 μl urea, 0.5% w/v CHAPS, 15 mM dithiothreitol, 0.2% w/v Pharma Lyte 3–10) for the 24-cm Immobiline Drystrips.

The protein spots were resolved using either a silver stain kit or SYPRO Ruby (Genomic Solutions, Markham, ON, Canada). The image of silver-stained gels was acquired using the Amersham Image Scanner, and that of the SYPRO Ruby gels was acquired using an Image-MASTER 2D Elite software (Amersham Biosciences, version 2003). Coomassie Blue R-250 was used to stain gels intended for use in mass spectrometry.

Values for each individual spot were determined using the Image Scanner used to acquire the two-dimensional gel images. This scanner was calibrated with a step wedge every 30 days, resulting in very accurate spot density determination. In addition, the fluorescent staining was also highly quantitative. This meant that two matched spots could be compared. The parameter that was most commonly used was spot-normalized volume. A difference map could be generated showing a colorimetric display of the differential protein expression in a two-dimensional experiment compared between the two paired gels. Parameters set used a minimum of 2-fold differential expression. Based on difference map comparisons among numerous hepatic ER two-dimensional preparations, the mandelic acid protein was identified as being upregulated in expression between chow-fed and fructose-fed hamster hepatic ER were identified. These protein spots were targeted for identification.

Protein spots that were stained either with SYPRO Ruby or with Coomassie Blue R-250 were used for in-gel trypsin digestion. The in-gel trypsin digestion and ZipTip C18 (Millipore, Ettobicoke, ON, Canada) sample purification followed the protocol described previously (9). Postsource decay-chemically assisted fragmentation (Amersham Bio-
RESULTS

Purity Determination of Hepatic ER Subcellular Fractionation—To conduct proteomics characterization of the ER compartment it was essential to obtain a highly pure hepatic ER fraction. To test the ER concentration and purity of the fractions, a protein assay, as well as immunoblotting for the ER marker proteins calnexin and ER60 was carried out (Fig. 1A). Antibodies against marker proteins for other cellular organelles were also used to test the purity of ER microsomes in the subcellular fractions. As can be seen in Fig. 1B, calnexin expression extended over most of the 20 ER fractions. This indicated that calnexin was present in both the heavier rough endoplasmic reticulum (RER) fractions, as well as the lighter smooth endoplasmic reticulum (SER) fractions, as described previously (10, 11). Based on calnexin protein expression in fractions 4–17 (the fractions used for two-dimensional analysis), we were able to obtain a 10-fold increase in ER enrichment over the whole cell lysate positive control. In keeping with previously published data, hepatic ER expression of ER60 was localized predominantly in the RER (Fig. 1B), as ER60 expression was observed in the heavier fractions (12). Based on ER60 protein expression, we were able to obtain a 16-fold increase in ER enrichment over the whole cell lysate positive control. Possible contamination of the hepatic ER fractions by other organelles was tested by immunoblotting (Fig. 1, B and C) using antibodies against catalese, a peroxisomal marker; 49K, a mitochondrial marker; 58K, a Golgi marker; LAMP-1, a lysosomal marker; and enolase, a cytoplasmic marker. The film exposures for these immunoblots were analyzed by densitometry, where the band intensity is represented as an integrated density value. There was no contamination detectable from other subcellular organelles, suggesting that very pure ER fractions could be isolated using this technique.

Hepatic ER Differential Protein Expression in Primary Hepatocytes from Chow- and Fructose-fed Hamster Livers—Proteomic analysis was initially performed by 7-cm pH 3–10 NL isoelectric focusing and minigel electrophoresis to test each newly produced ER subcellular fraction for quality of sample preparation prior to large scale proteomics analysis. This was followed by proteomic analysis using 24-cm pH 3–10 NL isoelectric focusing and 24 × 22-cm SDS-polyacrylamide gels of the hepatic ER. For the large scale hepatic ER proteomic analysis, two-dimensional sample preparations from three or four separate animals were pooled together for each experiment. These preparations were produced separately and only combined after the ER microsomal pellet resolubilization step described under “Experimental Procedures.” Because more than 20 animals were used in this portion of the study, this significantly reduced gel-to-gel variation caused by variation among individual animals. Eight pooled hepatic ER samples were produced, four for each treatment. Each sample, consisting of 200 μg of protein, was resolved on an analytical gel in duplicate or triplicate. Additional preparative gels consisting of 2 mg of protein were also performed to perform in-gel trypsin digestion for use in mass spectrometry. In total 26 large scale two-dimensional gels of hepatic ER were run.

Two-dimensional gels of hepatic ER isolated from primary hepatocytes of chow-fed (Fig. 2A) and fructose-fed (Fig. 2B) hamsters were analyzed and compared using the Amersham Biosciences Image Master 2D Elite software motif (version 2003). Differences in protein expression between the two treatments detected using this software are represented as a difference map (Fig. 2C). Fig. 2 is representative of 26 large scale two-dimensional gels. The difference map represents only proteins that were expressed more than 2-fold differentially between treatments. Shown on the gel are the spot numbers and most identities of the proteins that were discovered to be consistently expressed differentially between treatments. Eleven analytical large scale two-dimensional experiments were used to identify these 34 differentially expressed hepatic ER protein spots. The identity, expression information, and characteristics for the identified protein spots that were consistently expressed differentially are shown in Table I. Several of the 34 hepatic ER proteins detected could not be successfully identified as low abundance, and low molecular mass spots were difficult to identify. Even with 2 mg of protein loaded, these spots were not visible on the Coomassie R-250 preparative gels, and attempts to identify the SYPRO Ruby-stained spots were unsuccessful. However, some tryptic fragment profiles were obtained from these low abundance protein spots. The characteristics of these unidentified protein spots are shown in Table II. In addition, 17 hepatic ER protein spots that were not expressed differentially with the onset of insulin resistance were also identified by mass spectrometry and are represented in Fig. 2. The individual characteristics of these nondifferentially expressed proteins are given in Table III. These 17 protein spots constitute some of the more abundant proteins of the hepatic ER.

Hepatic ER One- and Two-dimensional Immunoblot for ER60: Differential Expression with the Onset of Insulin Resistance—To confirm the identification of seven isoforms of ER60...
in the hepatic ER all shown to be down-regulated at least 2-fold with insulin resistance, we performed immunoblotting analysis of two-dimensional gels from chow-fed and fructose-fed hepatic ER. Two-dimensional gel electrophoresis of control and insulin-resistant hepatic ER was carried out with 25 µg of hepatic ER protein, as described above, on 7-cm pH 3–10 nonlinear isoelectric focusing strips and 8% SDS-polyacrylamide gels. Immunoblotting was conducted as described above with an anti-ER60 primary antibody. Fig. 3, A and B, shows the ER60 two-dimensional gels analyzed using dedicated software. 34 hepatic ER protein spots were expressed differentially after 2 weeks of fructose feeding in this animal model. 22 of these proteins were identified. Protein spots that were stained either with SYPRO Ruby or with Coomassie Blue R-250 were used for in-gel trypsin digestion followed by mass spectrometric analysis (the Micromass MALDI-Q-TOF and the Amersham Biosciences MALDI-TOF Pro, with PSD capabilities). Database mining was performed using ProteinProspector MS-fit and MS-tag or the PROWl ProFound search engine, using a focused rodent or mammalian search. Sequence data, obtained by MALDI-TOF-PSD, was subjected to database searching using the PSD-Sonar database. In addition, 17 abundantly expressed hepatic ER proteins were identified and are also represented in the figure.

**DISCUSSION**

The fructose-fed hamster model of insulin resistance has proven invaluable in elucidating the mechanisms underlying carbohydrate-induced insulin resistance and the accompanying metabolic dyslipidemia (3). Evidence to date suggests that development of hepatic insulin resistance is a key underlying factor in the dysregulation of lipoprotein metabolism leading to metabolic dyslipidemia and an increased risk of atherosclerosis. Despite these important observations, a full characterization of the hepatic proteome and alterations caused by insulin resistance would be very useful in assessing global changes in protein expression underlying the phenotype observed in this animal model. We have used a proteomics approach to characterize hepatic ER proteins that are expressed differentially with fructose feeding and the onset of insulin resistance. A proteomic analysis of the ER-associated proteins was conducted so that protein species that would, in a whole cell proteome analysis, be below the detection limit or concealed by more abundant proteins, are now detectable on a two-dimensional map. The subcellular fractionation was first validated...
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through thorough testing of the ER fractions by immunoblot-ting with ER marker antibodies as well as markers against other potentially contaminating organelles. We employed the ER markers calnexin and ER60, which are known to be present in ribosome-laden rough RER as well as the SER (10, 11) and RER (12), respectively. We were able to obtain enrichment of 10- and 16-fold for calnexin and ER60, respectively, over whole cell lysate with no detectable contaminating proteins.

Large scale two-dimensional gel electrophoresis of hepatic ER was carried out, and reproducible two-dimensional profiles from both chow-fed and fructose-fed hepatic ER fractions were produced. Using difference maps from 11 large scale two-dimensional experiments that used either silver staining or the highly quantitative SYPRO Ruby stain, we identified 34 hepatic ER protein spots that were consistently expressed differentially with insulin resistance. 22 of these protein spots, corresponding to 13 different proteins, were positively identified.

| Protein spot no. | Protein identity | MALDI-TOF coverage % | MALDI-TOF fragment molecular mass kDa | Isoelectric point | Ratio control/fructose expression |
|------------------|------------------|-----------------------|--------------------------------------|------------------|----------------------------------|
| 2                | GRP94            | 10.7                  | 10.7                                  | 0.020            | ESI-Q-TOF sequence**              |
| 3                | GRP94            | 12.8                  | 12.8                                  | 0.004            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 4                | α-Glucosidase    | 14.2                  | 14.2                                  | 0.950            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 5                | Fibrinogen       | 16.7                  | 16.7                                  | 0.107            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 6                | Pglycoprotein    | 6.6                   | 6.6                                   | 0.747            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 7                | Catalase         | 23.7                  | 23.7                                  | 0.025            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 8                | ER60             | 21.2                  | 21.2                                  | 0.010            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 9                | ER60             | 29.3                  | 29.3                                  | 0.000            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 10               | ER60             | 37.6                  | 37.6                                  | 0.000            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 11               | ER60             | 34.3                  | 34.3                                  | 0.000            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 12               | ER60             | 38.8                  | 38.8                                  | 0.000            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 13               | ER60             | 36.2                  | 36.2                                  | 0.000            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 14               | ER60             | 30.1                  | 30.1                                  | 0.000            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 15               | ER60             | 29.3                  | 29.3                                  | 0.000            | KFAFQAEVNRM and KSIILVPTSAPRG      |
| 16               | PDI              | 20                    | 20                                    | 0.156            | KVIDAELFSFHSQKR and RELNFPSIH5FRQ   |
| 17               | PDI              | 20                    | 20                                    | 0.156            | KVIDAELFSFHSQKR and RELNFPSIH5FRQ   |
| 18               | PDI              | 20                    | 20                                    | 0.156            | KVIDAELFSFHSQKR and RELNFPSIH5FRQ   |
| 19               | Glutamate        | 15.4                  | 15.4                                  | 0.902            | KVIDAELFSFHSQKR and RELNFPSIH5FRQ   |
| 20               | ERp46            | 18.0                  | 18.0                                  | 0.202            | KVIDAELFSFHSQKR and RELNFPSIH5FRQ   |
| 21               | Apolipoprotein   | E                     | E                                     | 0.333            | KVIDAELFSFHSQKR and RELNFPSIH5FRQ   |
| 22               | ERp59            | 29                    | 29                                    | 0.000            | KVIDAELFSFHSQKR and RELNFPSIH5FRQ   |
| 23               | Peroxiredoxin    | 4                     | 4                                     | 0.000            | KVIDAELFSFHSQKR and RELNFPSIH5FRQ   |

* ESI, electrospray ionization.

TABLE II
Differentially expressed hepatic ER proteins after induction of insulin resistance with fructose feeding (not yet identified)

| Protein spot no. | MALDI-TOF fragments molecular mass kDa | Isoelectric point | Ratio control/fructose expression |
|------------------|---------------------------------------|------------------|----------------------------------|
| 1                | 774.492, 818.557, 862.55, 989.526, 1040.481, 1097.558, 1138.494, 1223.66, 1237.684, 1406.633, 1471.658, 1738.038, 1770.887, 2048.804, 2067.747, 2122.975 | 8.0 | 0.291771 |
| 12               | 567.338, 602.358, 694.364, 713.374, 722.381, 768.48, 856.578, 913.415, 1157.557, 1255.762, 1699.741, 1863.035, 2421.16, 2473.984, 2537.567, 3686.011, 3699.919, 3725.057 | 8.4 | 2.26776 |
| 26               | 26                                    | 8.2 | 0.546896 |
| 27               | 26                                    | 7.7 | Present only in control |
| 28               | 19                                    | 8.3 | 2.142587 |
| 29               | 20                                    | 7.9 | 0.38558 |
| 31               | 60                                    | 6.2 | 0.25 |
| 33               | 43                                    | 8.5 | 0.060516 |
| 34               | 35                                    | 8.4 | 0.101887 |
| 35               | 29                                    | 5.6 | 0.059524 |
the question of functional differences between isoforms. ER60, for instance, had seven isoforms that were down-regulated with insulin resistance, with at least two isoforms that did not meet our minimal 2-fold cut-off. It has been shown that proteins belonging to the thioredoxin family, including ER60, can be phosphorylated in the ER (13, 14). Identities of these differentially expressed hepatic ER proteins were confirmed by mass spectrometry several times in separate preparative gels thus validating their identity as the spot of interest and not simply a “miss-cut” protein spot.

Several ER chaperones were found to be expressed differentially with the onset of insulin resistance. The hepatic ER resident chaperone GRP94 (Gp96/endoplasm) (17–19) was found in our disease model to be up-regulated up to 15-fold over the control. On two-dimensional gels, GRP94 exhibited two distinct isoforms with the same molecular mass but slightly different isoelectric points. Interestingly, circulating (plasma) levels of GRP94 have also been shown to be up-regulated with type 1 diabetes (20). GRP94 has been shown to associate directly with apoB in the hepatic ER, and decreased expression was observed when apoB was depleted (21). In addition, GRP94 is a known ER stress protein (22), suggesting the ability of fructose feeding to induce ER stress. Three identified isoforms of protein disulfide isomerase (PDI) were found to be up-regulated between 2- and 3-fold with insulin resistance. PDI is part of the hepatic ER protein folding machinery, is known to be induced with ER stress, and its gene may be regulated by insulin (19, 22, 23). PDI was shown to be up-regulated with experimentally induced diabetes, an effect that was reversible with insulin (23, 24). PDI also constitutes one of the subunits of microsomal triglyceride transfer protein, an essential cofactor in apoB-containing lipoprotein maturation (23), which is also up-regulated with insulin resistance (5).

FIG. 3. Two-dimensional immunoblot analysis of ER60 in hepatic ER fractions of hepatocytes isolated from control and fructose-fed hamsters. 25 μg of protein from control (A) and fructose-fed (B) hamster hepatic ER were isoelectrically focused on 7-cm pH 3–10 nonlinear two-dimensional strips and subjected to electrophoresis in the second dimension on 10% SDS-polyacrylamide gels. These were transferred to polyvinylidene difluoride membrane and immunoblotted for ER60 expression. Inset, integrated density values of the ER60 protein spot for the two treatments. C. 20 μg of whole cell lysate from control and fructose-fed hamsters was subjected to electrophoresis on 8% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and immunoblotted for ER60. C is an immunoblot representative of multiple pairs of control and fructose-fed hepatic lysates for ER60 expression performed in triplicate. A significant down-regulation was evident for ER60 protein mass in the fructose-fed hepatocytes (p < 0.05, n = 4).

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| Protein spot no. | Protein identity | MALDI-TOF coverage | MALDI-TOF p value | Molecular mass actual/expected | pI actual/expected |
|------------------|------------------|---------------------|-------------------|------------------------------|-------------------|
| 35               | BiP              | 43.5                | 0.000             | 75/72.52                     | 5.05/1.1          |
| 36               | PDI              | 10.4                | 0.053             | 60/57.39                     | 4.83/4.8          |
| 37               | Calreticulin     | 39.6                | 0.000             | 55/48.40                     | 4.04/3.9          |
| 38               | ATPase           | 31.5                | 0.000             | 55/56.28                     | 5.05/2.2          |
| 39               | PDI              | 23.1                | 0.009             | 50/49.07                     | 5.05/0.0          |
| 40               | Nonmuscle actin  | 36.4                | 0.002             | 45/42.01                     | 5.35/3.5          |
| 41               | 40S ribosomal protein SA | 46.3            | 0.000             | 40/31.89                     | 4.84/8.4          |
| 42               | ER60             | 39.9                | 0.000             | 60/57.23                     | 6.26/0.0          |
| 43               | ER60             | 30.7                | 0.000             | 60/67.23                     | 6.06/0.0          |
| 44               | Aldolase B       | 33.8                | 0.000             | 40/39.97                     | 9.0/9.0           |
| 45               | Aldolase B       | 33.8                | 0.000             | 40/39.97                     | 9.2/9.0           |
| 46               | RACK1            | 71.3                | 0.000             | 35/35.52                     | 8.07/9.9          |
| 47               | Adenine nucleotide translocase | 12.7           | 0.281             | 33/34.59                     | 9.0/9.7           |
| 48               | Peroxiredoxin 4 | 35.9                | 0.001             | 31/31.22                     | 6.3/6.2           |
| 49               | Glutathione S-transferase | 29.4           | 0.050             | 26/25.55                     | 9.5/9.0           |
| 50               | Cytochrome b567  | 56.7                | 0.170             | 11/11.03                     | 5.05/1.1          |
| 51               | Retinol-binding protein 4 | 52.9         | 0.001             | 22/20.33                     | 6.5/5.7           |
Our current findings that seven isoforms of ER60 were down-regulated by an average of 2.7-fold in the fructose-fed hamster liver support these previous observations. This finding was validated further in our study using immunoblotting for ER60 on two-dimensional gels (Fig. 3). A recent proteomic analysis of the effect of obesity on hepatic protein expression showed ER60 was down-regulated in obese mice (30). Adeli et al. (31) showed a direct association of ER60 with apoB in HepG2 cells. When a lipid-poor state exists within the ER, the newly transcribed apoB is degraded via both proteasomal and nonproteasomal pathways. ER60 may be involved in the non-proteasomal pathway of apoB degradation (29). Therefore, with ER60 down-regulation in insulin resistance, apoB would be expected to have increased stability and would therefore increase VLDL production, potentially leading to dyslipidemia (3). It is important to note that multiple isoforms of ER60 were shown to be down-regulated in fructose-fed hamster liver. These isoforms were at the same molecular mass but slightly different isoelectric points, creating an ER60 "string" on the two-dimensional gels. The protein spots representing ER60 were all excised independently from the preparative gels and analyzed separately by mass spectrometry. The location of the ER60 isoform string was also determined by immunoblotting of two-dimensional gels with an ER60 antibody. Others have observed this ER60 isoform string in two-dimensional gels of whole liver proteome (32). These isoforms may represent different phosphoisoforms or different post-translational modifications of ER60.

The presence of thioredoxin domains appears to be a common feature of hepatic ER proteins that are down-regulated with insulin resistance because four of the five proteins identified here containing these domains were down-regulated with insulin resistance, with PDI being the exception. Thioredoxin domain-containing proteins may also be phosphorylated in the ER (13), a possible explanation for the multiple isoforms observed on our two-dimensional gels.

P-glycoprotein (MDR1) was found to be up-regulated by 2.5-fold in livers of fructose-fed hamster. P-glycoprotein functions in the intracellular transport of LDL cholesterol from the plasma membrane to the ER after receptor-mediated endocytosis (33). In experimentally induced diabetic rats, hepatic protein expression was increased more than 5-fold compared with controls, an effect that could be ameliorated with insulin administration (19). In another study, P-glycoprotein could be up-regulated by ER stress, an effect that could be partially reversed by antioxidant administration (34). Other functions of P-glycoproteins include the secretion of inflammatory components such as cytokines (35) and the translocation of cholesterol and phospholipids out of macrophages (35), both functions that lend themselves to an involvement in arteriosclerosis (35). Finally, P-glycoprotein gene expression has been shown to be induced by insulin (36).

α-Glucosidase was found to be up-regulated almost 2.5-fold in our animal model with the onset of insulin resistance. α-Glucosidase is a known hepatic ER protein that is essential for the secretion of hepatic lipase (37). α-Glucosidase is involved in the glucose, triglyceride, and hepatic lipase prior to its transport to the plasma membrane. Hepatic lipase mediates the conversion of LDL to VLDL (37). Increased small, dense LDL is characteristic of the atherogenic dyslipidemia associated with insulin-resistant states, and α-glucosidase inhibitors are currently used to treat diabetes (3, 37).

Fibrinogen, which has been implicated in inflammation leading to atherosclerosis with type 2 diabetes (38), is synthesized and secreted by hepatocytes (39). We found fibrinogen to be up-regulated 2-fold in insulin resistance. Rosiglitazone, a peroxisomal protein-activated receptor-y agonist, which is used to treat type 2 diabetes, has anti-inflammatory properties because it was shown to decrease fibrinogen levels (38). An elevated level of fibrinogen has also been shown to be associated with central obesity (40).

Glutamate dehydrogenase was shown to be down-regulated at least 6-fold in fructose-fed hamster liver. Glutamate dehydrogenase is a mitochondrial protein known to be involved in glucose metabolism (41) but has also been purified from rat hepatic ER (42). Glutamate dehydrogenase was shown to be down-regulated in the livers of fa/fa rats (41). In pancreatic β-cells, glutamate dehydrogenase is involved with glucose-induced insulin release and glucose homeostasis, with mutations in the gene resulting in hyperinsulinemia (43, 44).

Apoe was shown in this study to be up-regulated 3-fold in the hepatic ER of fructose-fed hamster liver. ApoE is a component of the mature VLDL particle, which is added during assembly in the hepatic ER. The discovery of hepatic ER apoE up-regulation with insulin resistance in this study helps to validate this method of protein characterization.

TAP1 is a hepatic ER protein that was shown in our study to be down-regulated 6.5-fold with insulin resistance. Mutations in this gene are commonly found in type 1 diabetics (45, 46). TAP1 translocates endogenous major histocompatibility complex (MHC) class I-binding peptides into the ER for assembly with MHC class I (47). Also, MHC class I up-regulation in hepatocytes occurs through TAP1 (48). ER60, calnexin, and TAP1 are known to be associated with one another (49).

Our proteomic analysis also included a number of abundant hepatic ER proteins whose identification validates our subcellular fractionation for ER isolation. 17 of the most abundant hepatic ER proteins were identified by mass spectrometry. All but 1 of these nonanalytical proteins is a known ER protein, further validating the efficiency of our hepatic ER subcellular fractionation protocol. In addition, the identification of these nonanalytical proteins further helps to complete our two-dimensional map of the hepatic ER. The abundant hepatic proteins identified in our analysis included PDI, ER60, peroxiredoxin 4, aldolase B, glutathione S-transferase, retinol-binding protein, cytochrome b5, nonmuscle actin, 40 S ribosomal protein SA, ATPase, BiP, and calereticulin (49–52). RACK1, which was shown not to be expressed differentially in the hepatic ER, is associated with the hepatic ER through its interaction with protein kinase C-β (63–69).

In summary, our study has yielded many new targets within the hepatic ER for future investigation. We have identified hepatic ER resident proteins, or transiently associated proteins, whose differential expression coincided with the onset of insulin resistance and may be implicated directly in hepatic complications of insulin resistance, insulin signaling attenuation, and/or lipoprotein dysregulation. Our current studies are focused on further characterization of these proteins and their potential involvement in the link between insulin resistance and metabolic dyslipidemia.

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Jean-Paul F. Morand, Joseph Macri and Khosrow Adeli

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