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Metabolic Labeling of Mammalian Organisms with Stable Isotopes for Quantitative Proteomic Analysis

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To quantify proteins on a global level from mammalian tissue, a method was developed to metabolically introduce ^{15}N stable isotopes into the proteins of *Rattus norvegicus* for use as internal standards. The long-term metabolic labeling of rats with a diet enriched in ^{15}N did not result in adverse health consequences. The average ^{15}N amino acid enrichments reflected the relative turnover rates in the different tissues and ranged from 74.3 mpe in brain to 92.2 mpe in plasma. Using the ^{15}N-enriched liver as a quantitative internal standard, changes in individual protein levels in response to cycloheximide treatment were measured for 310 proteins. These measurements revealed 127 proteins with altered protein level ($p < 0.05$). Most proteins with altered level have previously reported functions involving xenobiotic metabolism and protein-folding machinery of the endoplasmic reticulum. This approach is a powerful tool for the global quantitation of proteins, is capable of measuring proteome-wide changes in response to a drug, and will be useful for studying animal models of disease.

Quantitative mass spectrometry methods are typically based on the measurement of a compound relative to an internal standard. Therefore, a major goal in quantitative proteomics is to include a native internal standard for every protein in the sample to facilitate relative quantitative measurements.[1] Most methods derivatize one sample with a chemical tag containing only natural abundance isotopes and a second "control" sample with the identical tag containing specific atoms enriched in a "heavy" isotope.[2−4] These samples are mixed, and differences are estimated by the relative ion intensities of the two isotopomers. A limitation of this approach is that only selected functional groups of the molecule are tagged (e.g., thiols), and peptides without that functionality cannot be used in a comparative measurement. Furthermore, derivatizations are carried out in vitro after proteins have been fractionated and cannot account for errors and losses during any stage of the sample preparation prior to mixing.

An alternate strategy is the incorporation of stable isotope labeled atoms into metabolically synthesized proteins in vivo. These labeled cells are then added as an internal standard to cells grown in material with natural abundance isotopes at the beginning of the experiment to account for errors accrued during the sample preparation and measurement. This approach has been applied to microorganisms,[5−7] mammalian cells in culture,[8−10] and simple model organisms[11] where stable isotope labeled atoms (in the form of labeled nitrogen salts or amino acids) are included in the growth media. However, applications of stable isotope labeling in mammals have been limited to tracer amounts primarily because of the duration of the labeling period and possible adverse health consequences for the animal.[12,13]

We have developed a method to metabolically label mammalian organisms with ^{15}N to produce tissue-specific internal standards for global quantitative proteomic analyses of tissues. A labeling period of 44 days in a male rat resulted in a mean ^{15}N atomic enrichment of >90% in liver and plasma. These rat tissues provided an optimal source of tissue-specific internal standards to facilitate the quantitative proteomic analyses of complex mammalian tissue

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samples. Subsequent quantitative shotgun comparisons of rat liver lysates prepared from cycloheximide-treated and untreated animals using the ^{15}N liver standards reveal novel insight into global cellular responses to the reduction of protein synthesis using sublethal doses of the drug, cycloheximide.

EXPERIMENTAL SECTION

Materials. ^{15}N-Enriched (>99 atom % excess; ape) and unlabeled algal cells were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Enzymes were purchased from Roche Applied Science (Indianapolis, IN). The antibodies for BIP and PDI were purchased from Affinity BioReagents (Golden, CO) and Stressgen (Victoria, BC, Canada), respectively. Male Sprague−Dawley rats (3 weeks old) were purchased from Harlan (Indianapolis, IN). All methods involving animals were approved by the institutional Animal Research Committee and accredited by the American Association for Accreditation of Laboratory Animal Care.

Metabolic Labeling with ^{15}N-Labeled Algal Cells. *Rat Labeling.* Two three-week-old male Sprague−Dawley rats (from the same litter) were caged separately and maintained in a temperature-controlled (23 °C) facility with a 12-h light/dark cycle. Rats were fed specialized diets consisting of a protein-free rodent diet (Harlan Teklad, TD93328) supplemented with algal cells containing only natural abundance isotopes or algal cells enriched with >99 atomic percent excess (ape) ^{15}N (2 parts Harlan diet/1 part algae by mass). This mixture was offered to the rats for 30−45 min every 6 h (6 a.m., 12 p.m., 6 p.m., 12 a.m.) for 44 days. Rats had ad libitum access to water. Rats were weighed daily before the morning feeding to monitor growth.

Rat Growth. Six three-week-old male Sprague−Dawley rats (taken from the same litter) were caged separately in two groups of three. One group was fed standard rat chow ad libitum, and the other group was fed for 30−45 min every 6 h (6 a.m., 12 p.m., 6 p.m., 12 a.m.). All rats had ad libitum access to water. Rats were weighed daily before the morning feeding to monitor growth.

Tissue Harvest. Rats were anesthetized with mild Halothane inhalation, and blood was collected from the left ventricle into K3 EDTA Vacutainers (Becton Dickinson, Franklin Lakes, NJ). Uncoagulated blood was centrifuged to collect the plasma supernatant and remove the cellular components. Tissues (liver, duodenum, kidney, lung, heart, skeletal muscle, brain) were removed immediately, weighed, photographed, and placed on ice. Tissues were sliced into ~1−2-mm-thick slices and either fixed for microscopy or frozen in liquid N_2 and stored at −80 °C.

Gas Chromatography/Mass Spectrometry (GC/MS). Tissues (1 mg) were homogenized in 1 mL of H_2O manually using a Dounce homogenizer at 4 °C with 20−40 strokes, depending on the sample. The homogenates were microfuged at 14 000 rpm at 4 °C for 30 min. The supernatants were collected, and protein concentration was determined for the tissue supernatants and serum using Lowry H protein assay (Bio-Rad, Hercules, CA). Protein (1 mg) was precipitated from the supernatant using MeOH/$CHCl_3$.[14] The protein pellet was hydrolyzed by adding 1 mL of 6 M HCl, capping the vials, and heating at 110 °C for 24 h. The samples were dried under N_2 gas and then reconstituted in 1 mL of 1 M acetic acid. Each sample was vortexed and poured directly onto a disposable column containing 0.5 mL of washed cation-exchange resin (AG 50W-X8 100−200 mesh, hydrogen form, Bio-Rad Laboratories, Richmond, CA). The cation resin was washed twice with 5 mL of distilled water, and the bound material (including the amino acids) was eluted with 2 mL of 3 M NH_4OH into clean conical-bottom reaction vials. The vials were dried under N_2, and the resulting amino acids were derivatized to form the *tert*-butyl dimethyl silyl (tBDMS) derivatives as described previously.[15] All GC/MS measurements were made on a Hewlett-Packard model 5971A gas chromatograph/mass spectrometer (Palo Alto, CA) using electron ionization at 70 eV. The tBDMS-derivatized amino acids were separated on a DB-1 30 m × 0.25 mm × 0.25 μm film capillary column (J&W Scientific, Palo Alto, CA). The $[M - \textit{tert}\text{-butyl}]^+$ fragment ion was monitored for the unlabeled and ^{15}N-enriched isotopomers of alanine, glycine, valine, leucine, isoleucine, proline, serine, threonine, phenylalanine, aspartate/asparginine, and glutamate/glutamine. Likewise, the $[M - COOtBDMS]^+$ fragment ion was used for the measurement of methionine, histidine, and tyrosine. The integrated areas of the unlabeled and ^{15}N-labeled isotopomers were used to calculate the ^{15}N-amino acid enrichment using a least-squares analysis of the overlapping mass spectra.[16,17]

Quantitative Proteomic Analysis of Liver Lysates. *^{15}N-Labeled Liver Sample Preparation.* ^{15}N-Labeled liver slices were finely minced and homogenized (1 g/5 mL) in ice-cold homogenization buffer (100 mM K_2HPO_4/KH_2PO_4, pH 6.7, 5 mM $MgCl_2$, 250 mM sucrose) using 30 strokes in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at low speed (3000*g* for 15 min at 4 °C). The postnuclear supernatant (PNS) was collected and adjusted to 1 mg/mL with homogenization buffer for use as internal standards. Protein concentration was determined using the Lowry H protein assay (Bio-Rad). Taking the cost of the labeled algae into consideration, the resultant ^{15}N-labeled tissue usable as a quantitative internal standard is <$1/mg of protein.

Unlabeled Cycloheximide-Treated Liver Sample Preparation. Rats (40 adult males at ~250 g) were divided randomly into two groups of 20 rats and received either 50 mg/kg cycloheximide intraperitoneally (CHX) or no treatment (CTL) 4 h prior to sacrifice as described previously.[18] Livers were minced finely and homogenized (0.5 g/mL) in ice-cold homogenization buffer using a Polytron PT10/35 (Brinkmann, Westbury, NY) for 45 s with two passes from top to bottom. The homogenate was centrifuged at low speed (3000*g* for 15 min at 4 °C). The PNS fraction was collected and adjusted to 1 mg/mL with homogenization buffer for proteomic analysis. Protein concentrations were determined using the Lowry H protein assay (Bio-Rad). An aliquot of the ^{15}N-labeled PNS (250 μg) was added to an equivalent amount (250 μg) of each of the two samples (CTL and CHX PNS) as a quantitative internal standard. The mixed samples were diluted

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2-fold with 100 mM K_2HPO_4/KH_2PO_4, pH 6.7, and total membranes were pelleted by microfuging at 14 000 rpm for 15 min at 4 °C.

Immunoblots. SDS−PAGE was carried out using a 5−15% acrylamide gel and the Bio-Rad buffer system. For immunoblots, Immobilon-P filters were blocked for 1 h in Blotto (5% nonfat powdered milk/PBS/0.05% Tween-20). The filters were incubated overnight in primary antibody diluted 1:100 in Blotto. After three washes in Blotto, bound antibody was detected using [^{125}I]protein A. Bands on the immunoblots were visualized and quantified using a Typhoon 9400 (Amersham Biosciences).

Sample Digestion. To minimize any bias against integral membrane proteins, a high pH/proteinase K (hpPK) method described previously[19] was used to proteolytically digest the proteins in the mixed samples to peptides. Briefly, the total membrane pellet was resuspended at 1 mg/mL in 200 mM Na_2CO_3, pH 11, with five passes through an insulin syringe and incubated on ice for 1 h. The sample was then adjusted to 8 M urea, reduced, and alkylated as reported previously.[20] Proteinase K was added at a 1:50 enzyme/substrate ratio (w/w) and incubated at 37 °C for 5 h in a Thermomixer (Brinkmann, Westbury, NY). The reaction was quenched with formic acid (5% final concentration) and microfuged at 14 000 rpm at 4 °C for 15 min to remove insoluble particulates before analysis by multidimensional protein identification technology (MudPIT).

MudPIT. Protein digests were pressure-loaded onto a fused-silica capillary desalting column containing 5 cm of 5-μm Aqua C18 material (Phenomenex, Ventura, CA) and washed as described previously.[19] The desalted peptides were then eluted onto the back end of a triphasic chromatography column consisting of 7 cm of 5-μm Aqua C18 material (Phenomenex), 3 cm of 5-μm Partisphere strong cation exchanger (Whatman, Clifton, NJ), and 3 cm of 5-μm hydrophilic interaction chromatography material (PolyLC). The column was then placed in-line with a Surveyor quaternary HPLC pump (ThermoElectron, San Jose, CA) and analyzed using a 12-step separation described previously.[19] The HPLC pump was operated at a flow rate of 100 μL/min and was split to obtain flow through the column of ∼100−400 nL/min. As peptides eluted from the microcapillary column, they were electrosprayed directly into an LCQ-Deca mass spectrometer (ThermoElectron) with the application of a 2-kV spray voltage applied distally to the waste of the HPLC split as described by Martin et al.[21] A cycle of one full-scan mass spectrum (400−1400 m/z) followed by three data-dependent MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Application of the mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcaliber data system.

Data Analysis. *Peptide Sequence Identification.* Tandem mass spectra were analyzed twice using the database searching program SEQUEST-NORM,[22] creating two separate sets of output files stored in two separate directories. The first database search (standard) used a sequest.params file containing only a single static modification of +57 m/z on cysteine. The second database search (^{15}N-corrected) used a parameter file that contained a static modification on each amino acid, shifting the average mass to account for the enriched nitrogen atoms. All tandem mass spectra were searched against a fasta database containing all known and hypothetical *Rattus norvegicus* protein sequences downloaded from the National Center for Biotechnology Information on 2/28/2003. The program DTASelect[23] was used to select peptide sequences with normalized XCorr scores of >0.3 and a ΔCn of >0.1 from the SEQUEST-NORM output, assemble the peptides into protein identifications, and remove redundant protein identifications. To minimize false positives, only protein loci with two or more peptides exceeding the peptide filters were considered, resulting in an empirical confidence of >95%.[22]

Ion Chromatogram Extraction. For each peptide exceeding the DTASelect criteria in both the standard and ^{15}N-corrected database searches, ion chromatograms were extracted from the Xcaliber data file for the unlabeled and ^{15}N-enriched peptide isotope distributions using a modified version of EXTRACT-CHRO.[24] This version of EXTRACT-CHRO was written in Visual Basic 6 and included calls to a direct link library written in Visual C. The extraction program obtained the identified peptide sequences from the DTASelect-filter.txt output file and used these amino acid sequences to calculate the elemental composition and predict the isotope distribution[25] for each unlabeled and ^{15}N enriched peptide sequence—taking into account the ^{15}N atomic enrichment of the precursor and the resolution of the mass analyzer. Simply, the m/z range selected for each MS scan (precursor scan) was calculated to include the entire isotope distribution for the respective unlabeled and ^{15}N-enriched peptide pairs. Using this information, chromatograms were extracted for 100 MS scans surrounding the MS/MS spectrum that identified the peptide. The resulting ion chromatograms were stored in a tab-delimited file with a. chro extension. This approach is described in greater detail elsewhere.[24]

Calculation of Ion Current Ratios and Estimation of Protein Ratios. Each pair of ion chromatograms extracted from the Xcaliber data file was analyzed using a computer program called RelEx (for relative expression).[24] RelEx performed all aspects of the quantitative peak detection, peptide ratio calculations, omission of outliers, and estimation of the protein ratio. All protein ratios are normalized to actin to account for minor mixing errors between the two analyses (normalization factor, 0.98). Actin was chosen as a nonvarying protein because previous experiments under identical conditions, using alternative methodology, found actin levels to remain unchanged.[26]

Statistical Analysis. Differences between the unlabeled/^{15}N-enriched ratio of the control sample (sample CTL) and the unlabeled/^{15}N-enriched ratio of the cycloheximide sample (sample CHX) were assessed using the following approach. A weighted,

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pooled standard deviation (SD_{pooled}) was calculated from the RelEx protein ratio outputs from the two samples using the equation

$$SD_{pooled} = \sqrt{\frac{N_{CTL} - 1}{N_{CTL} + N_{CHX} - 2} SD_{CTL}^{2} + \frac{N_{CHX} - 1}{N_{CTL} + N_{CHX} - 2} SD_{CHX}^{2}} \quad (1)$$

where N_{CTL} and N_{CHX} are the number of ratio measurements in sample CTL and sample CHX, respectively. The SD_{pooled} was converted to a pooled standard error (SE_{pooled}) using the equation

$$SE_{pooled} = \sqrt{SD_{pooled}^{2}\left(\frac{1}{N_{CTL}} + \frac{1}{N_{CHX}}\right)} \quad (2)$$

Using this value and the mean ratios (av_{CTL} and av_{CHX}) between the two samples, a *t*-value was calculated using eq 3. The

$$t\text{-value} = \frac{|av_{CTL} - av_{CHX}|}{SE_{pooled}} \quad (3)$$

probability that the means were the same (*p*-value) was calculated from the *t*-value and the degrees of freedom ($df = N_{CTL} + N_{CHX} - 2$) using a two-tailed *t*-distribution. All calculations were performed in Microsoft Excel and only differences with *p*-values of <0.05 were considered significant.

RESULTS AND DISCUSSION

A three-week-old male Sprague−Dawley rat was metabolically labeled for 44 days by feeding with a specialized diet enriched in ^{15}N. This diet was formulated by supplementing a protein-free Harlan rodent diet with ^{15}N-enriched algal cells (>99 ape) included as the sole protein source. Because algae contains ~60−65% protein in its dry matter,[27] the two diet components were mixed at a 1:2 (algae mass/Harlan protein-free powder mass) ratio to result in ~20% protein content in the final diet.[28] A male littermate served as a control and was fed the same diet formulated with algae containing only natural abundance isotopes. The labeling period was from 3 to 9 weeks of age during which a maximal increase in body mass was expected for the weaned rats. During the labeling period, both the ^{15}N-enriched and unlabeled rat appeared normal (Supporting Information, Figure S-1). There were no overt differences in behavior, activity, or appearance between the two animals fed with the specialized diets or from other control animals fed with standard Harlan rodent diet. Both rats had similar increases in total body mass, 2.6-fold for the ^{15}N-enriched rat and 2.4-fold for the unlabeled rat (Figure 1). At the end of the labeling period, both rats were examined by in-house veterinarians and certified as "healthy". The relatively low increase in body mass was later shown in control animals to be attributed to the restricted feeding schedule rather than the specialized algae-containing diet (Figure S-2). Rats were sacrificed, and tissues (brain, liver, plasma, skeletal muscle, heart, kidney, lung, duodenum) were harvested and evaluated. Morphological assessment of tissues and cellular ultrastructure by standard histology and electron microscopy showed no visible abnormalities (data not shown). Therefore, it appeared that a ^{15}N-enriched diet had no adverse effect on the health of the animal.

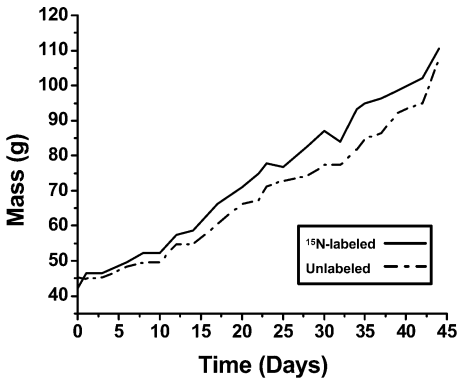

Figure 1. Mass gain of animals during the six-week labeling period. The labeled and unlabeled rat underwent similar mass increases during the six-week labeling period. Rats were weighed each morning, and their masses were plotted over time. The labeled rat (solid line) had a 2.6-fold increase in mass while the unlabeled rat (dashed line) had 2.4-fold.

To measure the degree of ^{15}N enrichment, we hydrolyzed the proteins extracted from each tissue to amino acids and measuring the respective ^{15}N amino acid enrichments by GC/MS. The average molar enrichments of the measured amino acids were used as an estimate of the atomic enrichment of the respective tissues. The average ^{15}N amino acid enrichments varied in the different tissues and ranged from lowest in brain (74.3 mpe) to highest in plasma (92.2 mpe) (Table 1, Figure S-3). This range is likely a function of the intracellular enrichment differences between amino acid precursor pools in the cells of each tissue. Although the nitrogen source from the diet is the same for all tissues, the amino acid precursor pool from which the tissue-specific proteins are synthesized is not. These pools are highly compartmentalized and dependent on both the transport of the labeled amino acid into the tissue cells and the dilution of the labeled amino acid intracellular precursor pool by both unlabeled and incompletely labeled amino acids released from protein breakdown.[29−31] Assuming that the transport of amino acids into cells is similar across different cell types, tissues with slower protein turnover will have an intracellular amino acid pool that equilibrates slower with the labeled atoms acquired in the animal's diet. Subsequently, because the enrichment of the amino acids with protein cannot exceed the enrichment of the amino acids it was synthesized from, a tissue with slower protein turnover will have a lower enrichment, not because the protein has not equilibrated with the amino acids it is being synthesized from but because the precursor has not equilibrated with the nitrogen in the diet. Thus, as expected, the differences in amino acid enrichment from hydrolyzed protein approximated the relative differences in the respective tissue's protein turnover.

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Table 1. Amino Acid Enrichments (Average ± SD) Measured by Gas Chromatography/Mass Spectrometry[a]

measured isotopomer	liver	plasma	brain	skeletal muscle	heart	lung	kidney	duodenum
^{15}N-alanine	91.32 ± 0.06	92.37 ± 0.04	74.74 ± 0.35	79.93 ± 0.11	81.40 ± 0.07	88.50 ± 0.11	89.50 ± 0.05	90.44 ± 0.02
^{15}N-glycine	87.36 ± 0.13	88.37 ± 0.11	68.82 ± 0.06	74.42 ± 0.09	76.35 ± 0.07	84.47 ± 0.07	85.13 ± 0.07	86.04 ± 0.04
^{15}N-valine	91.95 ± 0.03	92.89 ± 0.04	74.19 ± 0.08	80.09 ± 0.05	80.20 ± 0.05	88.77 ± 0.01	89.26 ± 0.02	90.52 ± 0.05
^{15}N-leucine	91.77 ± 0.02	93.67 ± 0.05	76.14 ± 0.07	79.49 ± 0.01	80.07 ± 0.06	88.46 ± 0.02	88.95 ± 0.04	90.31 ± 0.05
^{15}N-isoleucine	92.85 ± 0.03	92.75 ± 0.17	76.28 ± 0.05	79.97 ± 0.09	79.76 ± 0.09	89.61 ± 0.03	90.32 ± 0.02	90.71 ± 0.06
^{15}N-proline	91.73 ± 0.08	93.52 ± 0.15	66.62 ± 0.11	78.79 ± 0.09	80.86 ± 0.26	89.01 ± 0.08	88.82 ± 0.06	90.12 ± 0.04
^{15}N-methionine	92.59 ± 0.05	89.42 ± 0.02	75.74 ± 0.09	79.33 ± 0.13	81.71 ± 0.42	89.20 ± 0.17	90.75 ± 0.06	91.43 ± 0.20
^{15}N-serine	88.42 ± 0.02	93.67 ± 0.20	72.44 ± 0.12	77.50 ± 0.15	78.20 ± 0.17	86.50 ± 0.03	86.99 ± 0.03	88.18 ± 0.05
^{15}N-threonine	92.86 ± 0.19	93.02 ± 0.06	80.35 ± 0.30	82.57 ± 0.43	81.96 ± 0.15	90.33 ± 0.15	91.39 ± 0.15	91.28 ± 0.06
^{15}N-phenylalanine	91.91 ± 0.02	92.74 ± 0.02	78.85 ± 0.16	79.70 ± 0.04	78.56 ± 0.06	88.74 ± 0.05	89.62 ± 0.07	90.78 ± 0.05
^{15}N-Asx[b]	91.60 ± 0.04	92.19 ± 0.03	73.46 ± 0.11	78.02 ± 0.06	80.31 ± 0.08	88.43 ± 0.08	89.35 ± 0.03	90.65 ± 0.01
^{15}N-Glx[b]	91.56 ± 0.02	92.19 ± 0.03	75.27 ± 0.09	79.56 ± 0.09	80.67 ± 0.04	89.03 ± 0.06	89.44 ± 0.06	90.20 ± 0.03
$^{15}N_3$-histidine	86.53 ± 0.17	90.20 ± 0.16	66.85 ± 0.51	na[c]	76.09 ± 1.27	79.63 ± 0.47	82.24 ± 0.17	86.20 ± 0.40
^{15}N-tyrosine	92.51 ± 0.01	93.14 ± 0.14	80.32 ± 0.07	81.97 ± 0.06	80.32 ± 0.05	90.19 ± 0.04	91.22 ± 0.03	91.01 ± 0.08
average	91.07 ± 2.06	92.21 ± 1.66	74.29 ± 4.41	79.34 ± 2.01	79.75 ± 1.82	87.92 ± 2.81	88.79 ± 2.49	89.95 ± 1.75

[a] A graphical representation of these data can be found in Supporting Information, Figure S-3. [b] Asx represents the combined measurement of aspartate and asparagines; whereas, Glx represents the combined measurement of glutamate and glutamine. [c] Measurement not available because of the low intensity of the respective chromatographic peaks.

The true atomic percent excess of the labeled atoms must be known when material containing stable isotope enriched atoms is used as an internal standard. For example, the mean ^{15}N enrichment of liver protein is 91.1 ape, resulting in a broader isotope distribution for the labeled peptides that is shifted to lower m/z. Although the intensity of each individual isotope peak within the distribution is affected by the enrichment of the stable isotope labeled material, the sum of the intensities of the entire isotope distribution is not. Using RelEx, an m/z range is automatically chosen for the labeled and unlabeled peptide that incorporates the entire isotope distribution. Accounting for the incomplete atomic enrichment of the labeled peptide ensures an accurate prediction of the m/z range of the extracted ion chromatogram and enables the use of stable isotope labeled protein extracted from tissue of any enrichment as internal standard in proteomics.[24] A more thorough discussion is beyond the scope of this report and is described in detail elsewhere.[24]

The ^{15}N-enriched material was used as an internal standard to quantify the effect of cycloheximide on hepatic protein level. The drug cycloheximide inhibits the peptidyl transferase activity of the 60S ribosomal subunit in eukaryotic ribosomes and inhibits the elongation of nascent polypeptide chains during translation, making it a useful tool for blocking protein synthesis in eukaryotic cells.[32] Limited treatment with cycloheximide in cell culture is a classical approach for the termination of rapid turnover protein synthesis in eukaryotes and has been used to elucidate protein function by terminating cellular de novo synthesis for short periods of time.[32] Additionally, cycloheximide treatment of rats with sublethal doses has been shown to facilitate enrichment strategies for bona fide membrane proteins in fractionated secretory organelles by reducing the level of secretory proteins within the fraction.[18] To characterize any global cellular changes resulting from the use of this drug, a quantitative proteomics analysis was initiated to compare the relative levels of proteins between PNS prepared from cycloheximide-treated livers and PNS prepared from untreated livers.

The measurement of relative differences in protein level using stable isotope labeling requires that (1) the response between the unlabeled and labeled peptide in the mass spectrometer is equal to unity and (2) one of the two samples is a valid control for the other. Therefore, there exist two potential complications. First, although a majority of isotope ratio measurements have a one-to-one response in the mass spectrometer, selected methodologies have reported systematic errors.[33−35] Second, in the generation of the ^{15}N-enriched liver, the labeled rat was obtained from a separate litter and fed a nonstandard diet, making the sample a suboptimal control.

To minimize potential systematic errors, the ^{15}N-enriched tissue was used exclusively as an internal standard, rather than both an internal standard and control as compared with alternative quantitative proteomic analyses[2,8,9] (Figure 2). Peptide ratios were defined from the measured ion current ratios with signal/noise >3 using the quantitative proteomics algorithm RelEx and sorted by protein locus without any manual evaluation.[24] Peptide ratio outliers were omitted for proteins with ≤10 peptide ratios using a Dixon's Q-test.[36,37] Outliers were omitted for proteins with >10 peptide ratios if the individual value exceeded 2× the standard deviation of the mean. The remaining peptide ratios were then used to estimate the protein mean and standard deviation.

In measuring the effect of cycloheximide on protein level, ratios were determined for 1007 proteins in the ratio of cycloheximide-treated versus ^{15}N-enriched liver (Figure 2, ratio A) and 991 proteins in the ratio of untreated (control) versus ^{15}N-enriched liver (Figure 2, ratio B). Changes in protein level were then estimated by measuring the ratio of two ratios (ratio A/ratio B) for 310 proteins−protein ratios measured in both samples (Table S-1). By combining ratio A and ratio B, the contribution from the internal standard cancels, resulting in the ratio of cycloheximide-treated versus untreated tissue. Any systematic errors between

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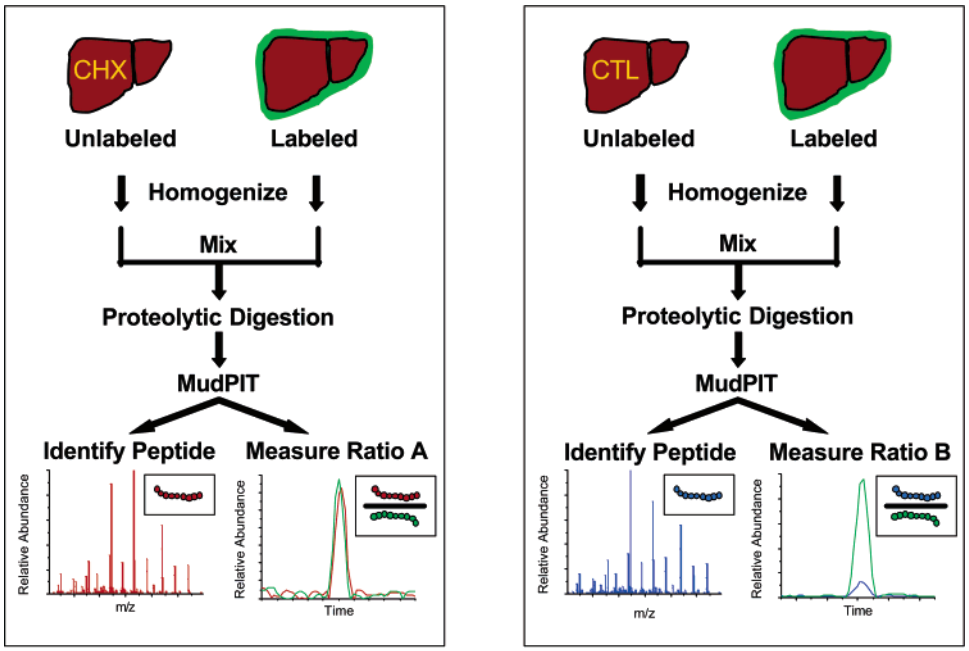

$$\frac{\text{Ratio A}}{\text{Ratio B}} = \frac{\text{CHX}}{\text{CTL}}$$

Figure 2. Quantitative proteomic analysis using metabolically labeled mammalian tissue as an internal standard. Untreated (CTL) and cycloheximide-treated (CHX) liver PNS and ^{15}N-enriched liver PNS (green highlight) were mixed early during the sample preparation to account for protein losses during the digestion and measurement (identification using tandem mass spectrum and measurement of ratio using mass chromatogram). Changes in protein level were estimated by measuring the ratio of two ratios using the following: (1) ratio A, the ratio of cycloheximide-treated (red peptide) vs ^{15}N-enriched liver (green peptide); and (2) ratio B, the ratio of untreated (blue peptide) vs ^{15}N-enriched liver (green peptide) By combining these two ratios, the internal standard (green) cancels resulting in the ratio of the untreated (red) vs cycloheximide-treated peptide (blue).

the labeled standard and unlabeled sample will occur in both ratios and will also cancel along with the internal standard—effectively minimizing any systematic errors (Figure 2).

Multiple peptides were used to calculate the relative abundance of each protein. This method is relatively precise with an average error of 25.3% RSD (12.2% RSE). As an example, individual extracted chromatograms used to calculate the protein ratio for BiP (Hspa5) are shown in Figure S-4. Of the measured mean protein ratios, 127 were statistically different ($p < 0.05$). Of these, over half (65 proteins) had >2 peptide measurements from both the CTL and CHX analyses and p-values of <0.01 (listed in Table 2). Because the hpPK method was used to produce peptides from the protein samples for MudPIT analysis, the bias against membrane proteins was minimized within these proteomic analyses.[19] Of the 65 proteins listed in Table 2, 55.4% were predicted transmembrane proteins using HMMTOP Version 2.0 (TMDs column in Table 2). Because the analyzed sample was an unfractionated liver lysate and the predicted proportion of membrane proteins in total mammalian genomes is ~25−35%,[38] the higher proportion of membrane proteins identified with altered protein levels between the two liver samples suggests potential functional significance.

The 65 proteins in Table 2 were grouped into known functional categories, and the result clearly revealed two major functional themes: (1) lipid oxidation and xenobiotic metabolism/detoxification for chemical defense and (2) quality control of misfolded newly synthesized proteins in the endoplasmic reticulum (ER). Most of the proteins in Table 2 had a measured increase in protein level in response to cycloheximide. One might hypothesize that an inhibitor of protein synthesis would reduce the level of most proteins. However, it is clear that protein level is controlled by a delicate balance between both protein synthesis and breakdown.[29] Thus, inhibition of protein synthesis can only decrease the level of a protein if the respective protein degradation machinery remains unchanged. The measured increase in the level of most of the proteins involved in xenobiotic metabolism and quality control in the ER suggests that the degradation of these proteins may be inhibited in parallel to increase the level of the appropriate proteins and counter the effects of the cycloheximide.

The cytochrome P450 family of enzymes represent a group of heme−thiolate monooxygenases found in the endoplasmic reticulum that plays a major role in the metabolism of xenobiotics and endogenous lipid-soluble substrates. This enzyme system takes lipid-soluble substrates and converts them to more water-soluble products, which can then be excreted in urine and exhibit reduced pharmacological/biological activity.[39] Hundreds of cytochrome P450 enzymes have been identified,[40] and many exist as heterogeneous complexes, some with known altered catalytic function.[39]

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Table 2. Proteins with Measured Differences between the Cycloheximide-Treated and Untreated Tissue Relative to Actin ($p < 0.01$) Grouped into Functional Categories[a]

	accession number[b]	Swiss-Prot AC[c]	gene locus	enzyme class	TMDs[d]	description	untreated av ratio	untreated SD	untreated n[e]	cycloheximide-treated av ratio	cycloheximide-treated SD	cycloheximide-treated n[e]	factor change[f]	p-value
						Lipid/Cholesterol/Steroid Oxidation and Metabolism								
1	gi|21955148|ref|NP_665725.1|	Q64581	Cyp3a18	EC 1.14.14.1	4 in	cytochrome P450, 3A18	1.18	0.277	17	0.66	0.092	18	−1.78	<0.0001
2	gi|9506529|ref|NP_062057.1|	P08683	Cyp2c11	EC 1.14.14.1	3 in	cytochrome P450, 2C11	1.76	0.339	26	3.50	1.001	43	1.99	<0.0001
3	gi|13929204|ref|NP_114027.1|	P24470	Cyp2c23	EC 1.14.14.1	0 out	cytochrome P450, 2C23	1.12	0.305	13	1.62	0.346	12	1.44	0.0009
4	gi|6978747|ref|NP_036862.1|	P10634	Cyp2d2	EC 1.14.14.1	1 in	cytochrome P450, 2D2	1.32	0.523	63	1.82	0.594	27	1.38	0.0001
5	gi|27694244|ref|XP_214015.1|	P09875	Ugt2b1	EC 2.4.1.17	2 in	UDP-glucuronosyltransferase 2B1 precursor, microsomal	0.47	0.072	17	0.69	0.099	10	1.45	<0.0001
6	gi|23463309|ref|NP_695226.1|	P08542	Ugt2b3	EC 2.4.1.17	1 out	UDP-glucuronosyltransferase 2B3 precursor, microsomal	0.79	0.091	6	1.30	0.212	7	1.64	0.0002
7	gi|14010881|ref|NP_114186.1|	P36511	Ugt2b12	EC 2.4.1.17	1 out	UDP-glucuronosyltransferase 2B12 precursor, microsomal	1.12	0.300	18	1.46	0.293	13	1.30	0.0038
8	gi|25742739|ref|NP_036952.1|	P18163	Facl2	EC 6.2.1.3	1 in	fatty acid coenzyme A ligase, long chain 2	0.68	0.098	6	1.18	0.199	15	1.74	<0.0001
9	gi|16758398|ref|NP_446059.1|	O88813	Facl5	EC 6.2.1.3	1 in	fatty acid coenzyme A ligase, long chain 5	0.42	0.085	9	0.69	0.107	31	1.65	<0.0001
10	gi|13162326|ref|NP_077057.1|	Q9ES38	none	na	1 in	bile acid CoA ligase	0.59	0.150	12	0.84	0.208	18	1.43	0.0012
11	gi|6981594|ref|NP_037015.1|	P49889	Ste	EC 2.8.2.4	0 out	estrogen sulfotransferase	4.11	0.663	5	7.05	0.927	3	1.72	0.0019
12	gi|6981598|ref|NP_036793.1|	P15589	Sts	EC 3.1.6.2	2 out	steroid sulfatase	0.98	0.188	22	1.51	0.303	30	1.54	<0.0001
13	gi|20302049|ref|NP_620232.1|	P20070	Dia1	EC 1.6.2.2	0 out	NADH-cytochrome *b*-5 reductase	0.69	0.114	12	0.96	0.213	15	1.39	0.0007
14	gi|13399338|ref|NP_085075.1|	P04166	Omb5	na	1 in	cytochrome *b*-5 outer mitochondrial membrane isoform	0.65	0.056	4	1.02	0.103	4	1.57	0.0007
15	gi|34869959|ref|XP_216452.1|	Q8VCH6	Dhcr24	na	3 in	24-dehydrocholesterol reductase	0.54	0.049	17	0.84	0.206	6	1.55	<0.0001
16	gi|11693158|ref|NP_071784.1|	Q9Z2Z8	Dhcr7	EC 1.3.1.21	9 out	7-dehydrocholesterol reductase	0.33	0.051	9	0.51	0.113	20	1.57	0.0001
17	gi|17105350|ref|NP_476545.1|	Q64591	Decr1	EC 1.3.1.34	0 out	2,4-dienoyl CoA reductase 1, mitochondrial	0.27	0.063	17	0.20	0.098	31	−1.40	0.0049
18	gi|27705988|ref|XP_228274.1|	Q8CHN6	Spl	EC 4.1.2.27	0 out	sphingosine-1-phosphate lyase	0.62	0.056	13	1.05	0.228	9	1.71	<0.0001
19	gi|8392839|ref|NP_058683.1|	P16638	Acly	EC 2.3.3.8	0 out	ATP-citrate lyase	0.15	0.068	8	0.27	0.059	11	1.83	0.0005
20	gi|6978813|ref|NP_036976.1|	P07687	Ephx1	EC 3.3.2.3	0 out	epoxide hydrolase 1	0.60	0.106	27	0.84	0.141	20	1.41	<0.0001
21	gi|13591981|ref|NP_112311.1|	Q62811	none	na	2 out	oxidosqualene cyclase	0.42	0.054	6	0.56	0.030	4	1.34	0.0015
22	gi|8392962|ref|NP_058996.1|	Q63276	none	na	2 out	Kan-1; bile acid-CoA-amino acid *N*-acyltransferase	0.59	0.276	7	1.14	0.371	12	1.93	0.0033
						Endoplasmic Reticulum								
23[g]	gi|6981486|ref|NP_037199.1|	P07153	Rpn1	EC 2.4.1.119	2 in	dolichyl-diphosphooligosaccharide protein glycotransferase 67 kDa subunit	0.58	0.149	17	1.08	0.182	11	1.88	<0.0001
24	gi|13928974|ref|NP_113886.1|	P25235	Rpn2	EC 2.4.1.119	3 out	dolichyl-diphosphooligosaccharide protein glycotransferase 63 kDa subunit	0.82	0.123	11	1.10	0.258	8	1.35	0.0050
25	gi|27716341|ref|XP_233581.1|	O54734 mouse	LOC313648	EC 2.4.1.119	2 in	dolichyl-diphosphooligosaccharide protein glycotransferase 48 kDa subunit	0.68	0.177	6	1.10	0.145	6	1.62	0.0011
26[g]	gi|25742763|ref|NP_037215.1|	P06761	Hspa5	na	1 out	78 kDa glucose-regulated protein (BiP)	0.90	0.282	14	1.18	0.107	12	1.31	0.0035
27	gi|25282419|ref|NP_742005.1|	P35565	Canx	na	1 out	calnexin	0.89	0.096	13	1.46	0.062	6	1.64	<0.0001
28	gi|11693172|ref|NP_071794.1|	P18418	Calr	na	1 out	calreticulin	0.98	0.154	10	1.65	0.359	8	1.69	0.0001
29[g]	gi|6981324|ref|NP_037130.1|	P04785	P4hb	EC 5.3.4.1	0 out	protein disulfide isomerase (PDI)	0.74	0.202	12	1.37	0.301	16	1.85	<0.0001
30	gi|17865351|ref|NP_446316.1|	P55072 human	Vcp	na	0 out	transitional endoplasmic reticulum ATPase	1.68	0.335	57	2.68	0.727	26	1.60	<0.0001
31	gi|21717659|ref|NP_579829.1|	P16303	Ces10	EC 3.1.1.1	0 out	liver carboxylesterase 10	1.35	0.536	10	1.99	0.539	17	1.48	0.0060
32	gi|13592047|ref|NP_112359.1|	O09175	Rnpep	EC 3.4.11.6	1 in	aminopeptidase B (*R. norvegicus*)	0.57	0.080	12	1.14	0.436	5	2.02	0.0003
33	gi|20302024|ref|NP_620222.1|	Q63617	Orp150	na	0 in	150 kDa oxygen-regulated protein	0.85	0.082	11	1.50	0.262	17	1.77	<0.0001
34	gi|13994184|ref|NP_113937.1|	O88941	none	na	1 in	glucosidase 1	0.65	0.122	8	0.93	0.172	4	1.44	0.0077
35	gi|19705453|ref|NP_599176.1|	P08011	Mgst1	EC 2.5.1.18	3 out	microsomal glutathione *S*-transferase 1	0.74	0.076	17	1.24	0.146	22	1.66	<0.0001
36	gi|16758274|ref|NP_445964.1|	Q9Z0V5	PrxIV	na	1 in	peroxiredoxin 4	0.93	0.200	26	1.35	0.367	25	1.46	<0.0001
37	gi|34871082|ref|XP_220576.1|	Q99J12	Flana	na	3 out	hypothetical protein similar to carcinoma-related gene	0.75	0.133	6	1.11	0.140	5	1.48	0.0019
						Translation								
38	gi|8394364|ref|NP_058895.1|	Q07984	Ssr4	na	2 in	translocon-associated protein, δ subunit	0.50	0.049	5	0.88	0.114	9	1.75	<0.0001
39	gi|34852367|ref|XP_215371.1|	P56554	Ube2g2	EC 6.3.2.19	1 out	similar to ubiquitin conjugating enzyme E2G 2	0.69	0.155	3	1.49	0.134	2	2.16	0.0097
40	gi|27681617|ref|XP_225097.1|	P20001	Eef1a1	na	0 out	elongation factor 1, α 1	0.44	0.047	7	0.63	0.053	7	1.45	<0.0001
41	gi|15805031|ref|NP_284925.1|	Q64718	Eef1a2	na	0 out	elongation factor 1, α 2	0.34	0.089	13	0.48	0.094	9	1.40	0.0024
42	gi|8393296|ref|NP_058941.1|	P05197	Eef2	na	0 out	elongation factor 2	0.35	0.083	6	0.77	0.160	12	2.19	<0.0001
43	gi|27672918|ref|XP_213364.1|	P04765	Eif4a1	na	0 out	eukaryotic initiation factor 4A−I	0.48	0.054	16	0.65	0.121	9	1.37	<0.0001
44	gi|13592067|ref|NP_112370.1|	P29314	Rps9	na	0 in	40S ribosomal protein S9	0.47	0.083	11	0.65	0.047	3	1.38	0.0046
45	gi|8394009|ref|NP_058797.1|	P10111	Ppia	EC 5.2.1.8	0 out	peptidyl-prolyl cis−trans isomerase A	0.49	0.054	6	0.69	0.082	5	1.43	0.0007

Table 2. (Continued)

	accession number[b]	Swiss-Prot AC[c]	gene locus	enzyme class	TMDs[d]	description	untreated av ratio	untreated SD	untreated n^e	cycloheximide-treated av ratio	cycloheximide-treated SD	cycloheximide-treated n^e	factor change[f]	p-value
						Metabolic Enzymes								
46	gi|6981002|ref|NP_037221.1|	P17625	Gys2	EC 2.4.1.11	0 out	glycogen synthase, liver	0.56	0.043	3	0.22	0.062	2	−2.61	0.0048
47	gi|11560087|ref|NP_071604.1|	P09811	Pygl	EC 2.4.1.1	0 out	glycogen phosphorylase, liver	0.66	0.133	8	0.37	0.200	14	−1.79	0.0016
48	gi|27695604|ref|XP_215697.1|	Q8CE68 mouse	LOC295399	na	0 out	similar to amylo-1,6-glucosidase, 4-α-glucanotransferase isoform 1	1.18	0.232	7	0.62	0.143	4	−1.92	0.0019
49	gi|13540663|ref|NP_110477.1|	O09171	Bhmt	EC 2.1.1.5	1 in	betaine-homocysteine methyltransferase	0.80	0.148	28	1.34	0.276	38	1.67	<0.0001
50	gi|31982384|ref|NP_062159.1|	Q8K571	none	na	0 out	alcohol dehydrogenase 1	0.40	0.058	10	0.55	0.108	10	1.38	0.0011
51	gi|15100179|ref|NP_150238.1|	O88989	Mdh	EC 1.1.1.37	1 in	malate dehydrogenase 1	0.27	0.046	13	0.39	0.100	25	1.43	0.0003
52	gi|8392920|ref|NP_058830.1|	P07824	Arg1	EC 3.5.3.1	1 in	arginase 1, liver	0.60	0.088	13	0.89	0.101	5	1.48	<0.0001
53	gi|25453414|ref|NP_037289.1|	P09034	Ass	EC 6.3.4.5	1 in	arginosuccinate synthetase 1	0.47	0.141	11	0.67	0.105	10	1.43	0.0016
54	gi|8393349|ref|NP_058877.1|	P25093	Fah	EC 3.7.1.2	0 out	fumarylacetoacetate hydrolase	0.62	0.095	22	0.97	0.164	18	1.57	<0.0001
55	gi|8393557|ref|NP_058929.1|	O88655	Hppd	na	0 out	4-hydroxyphenylpyruvic acid dioxygenase	0.69	0.098	11	1.03	0.167	22	1.49	<0.0001
56	gi|27674996|ref|XP_214155.1|	Q9D8C9 mouse	LOC297713	na	0 out	similar to purine nucleoside phosphorylase (PNP)	0.76	0.091	11	1.20	0.312	6	1.58	0.0005
57	gi|9506447|ref|NP_062165.1|	P14141	Ca3	EC 4.2.1.1	0 out	carbonic anhydrase 3	1.99	0.789	30	3.46	1.772	12	1.73	0.0006
						Peroxisome								
58	gi|16758056|ref|NP_445791.1|	Q63448	Acox3	EC 1.3.3.6	3 in	acyl-coenzyme A oxidase 3, peroxisomal	0.56	0.074	8	0.89	0.131	5	1.58	0.0001
59	gi|19424318|ref|NP_598290.1|	P07896	Ehhadh	EC 4.2.1.17, EC 5.3.3.8, EC 1.1.1.35	4 in	peroxisomal bifunctional enzyme	0.17	0.029	10	0.22	0.034	11	1.34	0.0006
60	gi|6978607|ref|NP_036652.1|	P04762	Cat	EC 1.11.1.6	0 out	catalase	0.47	0.087	26	0.87	0.179	30	1.84	<0.0001
						Phosphatase								
61	gi|14861868|ref|NP_149090.1|	Q64612	Esp	EC 3.1.3.48	2 out	protein tyrosine phosphatase, receptor type, W	0.50	0.377	6	1.26	0.251	5	2.52	0.0041
						Transport								
62	gi|16758646|ref|NP_446253.1|	Q9Z1J8	Sec14l3	na	0 out	SEC14-like protein 3	0.60	0.106	21	0.94	0.100	9	1.55	<0.0001
						Nucleus								
63	gi|12025524|ref|NP_072169.1|	Q64677	none	na	0 in	histone H2B	1.29	0.277	10	0.75	0.026	4	−1.73	0.0024
						Apoptosis								
64	gi|19424244|ref|NP_598245.1|	Q9JLG5	Brp44l	na	2 in	brain protein 44-like (*R. norvegicus*)	0.44	0.043	3	0.25	0.045	5	−1.73	0.0012
						Serum Protein								
65	gi|27678774|ref|XP_215033.1|	P11517	none	na	0 out	hemoglobin β chain, minor form	0.30	0.080	14	0.50	0.124	12	1.64	0.0001

[a] Protein ratios were determined automatically using RelEx.[24] [Note: Protein ratios were determined for the untreated-control vs ^{15}N-enriched (991 protein ratios) and the cycloheximide-treated vs ^{15}N-enriched liver samples (1007 protein ratios). The factor change was determined using a ratio of 2 ratios for the 310 proteins detected in both samples (Table S-1). Of the 310 total ratios measured, 127 had means that were different between the CTL and CHX analyses ($p < 0.05$).] [b] National Center for Biotechnology Information (NCBI) Accession Number. [c] Swiss Prot Accession Number. [d] Prediction of transmembrane domains using HMMTOP (Version 2.0). The number represents the number of predicted transmembrane domains and the "in" and "out" represent the predicted localization of the N-terminus. [e] Number of independent measurements of the protein ratio. [f] All changes are normalized relative to actin. [g] Quantitative Western data available in Figure 3.

Four cytochrome P450 enzymes belonging to three subfamilies (3A, 2C, 2D) were identified with altered ratios (proteins 1−4, Table 2). Interestingly, both 2C (2C11, 2C23) and the 2D (2D2) cytochromes increased in level while the 3A18 decreased. Because there are hundreds of cytochrome P450 enzymes present in each cell, these results suggest that differential modulation of multiple cytochrome levels may facilitate a fine-tune response for the elimination of specific types of drugs. Because sequence similarity is high between family members (the 3A cytochromes have 67.1% sequence identity), only peptides that uniquely identified each respective protein were used for the relative ratio calculation. The ability to distinguish between homologous members of this enzyme class illustrates the advantages of using a metabolic labeling strategy for quantitative proteomics because comparisons are not limited to peptides containing specific residues.

In addition to the cytochrome P450 family, xenobiotics are also converted to polar and water-soluble intermediates by other classes of enzymes. There are multiple examples listed in Table 2 that exhibit this theme, all of which have a measurable increase in protein level with cycloheximide treatment. For example, glucuronosyltransferases and members of the sulfotransferase and sulfatase enzymes families are involved in the elimination of lipid-soluble substrates by the conjugation of glucuronosyl and sulfate, respectively.[41,42] Three members of the glucuronosyltransferase family (proteins 5−7) and a sulfotransferase and sulfatase (proteins 11 and 12) are listed. Additionally, three members of the fatty acid coenzyme A ligase family were shown to have an induced level. These enzymes are involved in the degradation of lipids by β elimination (proteins 8−10).[43] Collectively from these results, we conclude that sublethal treatment with cycloheximide results

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in a more global effect than just the termination of protein synthesis.

One consequence of protein synthesis termination is the accumulation of incomplete polypeptide chains and partially assembled protein complexes. The ER has a quality-control (QC) system for monitoring the folding of newly synthesized proteins. Only correctly folded or "native" conformers are allowed to reach their final destinations.[44] If the folding and maturation process fails, the protein is retained and eventually degraded. Sensors of this QC system include molecular chaperones that bind to incompletely folded proteins to stabilize them and assist with the folding process.

Interestingly, seven proteins involved in quality control were found at increased levels (proteins 23−29). The 78-kDa glucose-regulated protein (also known as BiP, protein 26) binds to nascent and newly synthesized proteins and assists in their folding and ER-associated degradation through the unfolded protein response.[44−46] Calnexin and calreticulin (proteins 27 and 28) are two lectin chaperones that assist in the folding of proteins modified with monoglucosylated N-linked glycans, and protein disulfide isomerase catalyzes the oxidation, isomerization, and reduction of disulfide bonds.[44] UDP-glycosyltransferase regulates the association of glycoproteins with calnexin or calreticulin by the reglycosylation of the glycoproteins for another opportunity to achieve native conformation. Because the transfer of N-linked oligosaccharide chains has been shown to contribute to protein stability, it was interesting to observe that the level of oligosaccharyltransferase, the key enzyme that catalyzes the transfer of high-mannose sugar $GlcNAc_2$-Man_9-$Gluc_3$ from lipid-linked oligosaccharide donors to asparagine acceptor sites on nascent polypeptides, also increased. In fact, this enzyme complex is composed of three subunits (ribophorin I/the 67-kDa subunit, ribophorin II/the 63-kDa subunit, and the 48-kDa subunit), and all three subunits have a coordinated increase in Table 2 (proteins 23−25). Collectively, the increased levels of individual QC components reflect a global cellular response to prematurely terminated polypeptides and irregularities in protein complex formation.

As a validation our quantitative mass spectrometry based strategy using metabolically labeled tissue, three of the proteins involved in QC were selected for confirmation using an alternative analysis (Table 2). A quantitative Western analysis was conducted using antibodies against Hspa5 (BiP, protein 26), P4hb (PDI, protein 29), and Rpn1 (ribophorin I, protein 23) to determine the relative abundance of these proteins in the control and cycloheximide-treated samples (Figure 3). All three proteins exhibit similar relative changes between CHX and CTL as measured using the metabolically labeled tissue and mass spectrometry (Table 2). In addition to the Western analysis shown in Figure 3, BiP was quantified in four separate analyses to estimate the relative precision of the assay (additional Westerns not shown). The resulting BiP ratio by Western blot was 1.53 ± 0.39 (mean ± SD). In comparison, the BiP ratio by mass spectrometry was 1.31 ± 0.11, which is not statistically different from the mean ratio measured by Western blot ($p > 0.10$).

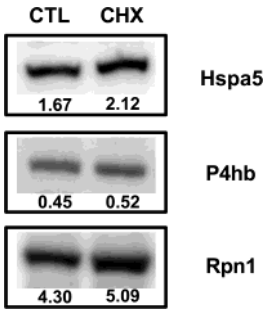

Figure 3. Quantitation of protein ratios between the cycloheximide-treated and untreated tissue using quantitative Western analysis. Equal protein loads (25 μg) of untreated (CTL) and cycloheximide-treated (CHX) liver homogenates were resolved on SDS−PAGE (5−15%) and transferred onto Immobilon-P for Western analysis. All antibodies were used at 1:100 dilution. Detection was by Typhoon analysis after incubation with [^{125}I]protein A.

In conclusion, this study metabolically labeled a male rat with stable isotope labeled material and used the labeled liver tissue as an internal standard for the relative quantitation of proteins using mass spectrometry. The effect of the drug cycloheximide on individual protein level was quantified for 310 different proteins in a single proteomic experiment. There are countless possibilities for the application of this labeling strategy for comparative proteomic analyses of mammalian tissues. Comparative assays monitoring changes in protein expression can be applied to the molecular characterization of transgenic animal tissues that are not detectable by the standard histological evaluations. Diseased and healthy tissues can be compared directly in mammalian model organisms for the elucidation of disease mechanisms and the identification of novel drug targets. Furthermore, because clinically important pharmacokinetic drug interactions can occur when one drug modulates the level of cellular metabolic enzymes, coadministered drugs necessitate the ability to quantitatively monitor the induction of drug-metabolizing enzymes. Characterization of these effects can aid in the selection of noninducers for clinical development. Finally, future directions for the standardization of this strategy could include large-scale tissue banking resource centers to distribute well-characterized labeled tissues for quantitative proteomic analyses.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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