Warm Ischemia-induced Alterations in Oxidative and Inflammatory Proteins in Hepatic Kupffer Cells in Rats*

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The aim of the study was to investigate the impact of ischemia/reperfusion injury on the proteome of Kupffer cells. Lean Zucker rats ($n = 6$ each group) were randomized to 75 min of warm ischemia or sham operation. After reperfusion for 8 h, Kupffer cells were isolated by enzymatic perfusion and density gradient centrifugation. Proteins were tryptically digested into peptides and differentially labeled with iTRAQ (isobaric tags for relative and absolute quantitation) reagent. After fractionation by cation exchange chromatography, peptides were identified by mass spectrometry (ESI-LC-MS/MS). Spectra were interrogated against the Swiss-Prot database and quantified using ProteinProspector®. The results for heat shock protein 70 and myeloperoxidase were validated by ELISA. Quantitative information for more than 1559 proteins was obtained. In the ischemia group proteins involved in inflammation were significantly up-regulated. The ratio for calgranulin B in the ischemia/sham group was 1.81 ± 0.97 ($p < 0.0001$), for complement C3 the ratio was 1.81 ± 0.49 ($p < 0.0001$), and for myeloperoxidase the ratio was 1.30 ± 0.32. Myeloperoxidase was only recently documented in Kupffer cells. The antioxidative proteins Cu,Zn-superoxide dismutase (1.34 ± 0.19; $p < 0.001$) and catalase (1.23 ± 0.43; $p < 0.001$) were also elevated. In conclusion, ischemia/reperfusion injury induces alterations in the Kupffer cell proteome. Isotope ratio mass spectrometry is a powerful tool to investigate these reactions. The ability to simultaneously monitor several pathways involved in reperfusion stress may result in important mechanistic insight and possibly new treatment options. ***Molecular & Cellular Proteomics 5:979–986, 2006.***

There is substantial evidence that indicates that Kupffer cells play a central role in the pathogenesis of liver parenchymal cell damage as seen during ischemia/reperfusion injury (1). The activation of Kupffer cells that occurs during ischemia results in the production of oxygen radicals and the modulation of proinflammatory and anti-inflammatory cytokines (2). These mediators are implicated in hepatic cell injury and sinusoidal function. It has been demonstrated that modulation of Kupffer cells can protect the liver (3, 4). The mechanisms that have been postulated include a decreased production of oxygen radicals and neutrophil infiltration. However, little is known about the differential effects of protein expression and changes in post-translational modifications in Kupffer cells that are exposed to warm ischemia. One objective of proteome research is to identify and describe the complex responses of an organism to different stimuli (5, 6). Rapid progress in both protein separation and identification techniques has made mass spectrometry a powerful tool for characterizing the protein content of a biological system (7–13).

Isotope ratio mass spectrometry with isobaric tags for relative and absolute quantitation (iTRAQ)[1] allows for the quantification and identification of the protein content of up to four samples in one experiment by labeling all peptides in a given sample (14). Peptides of the samples are modified by incorporating the four different reagents. The derivatized peptides are indistinguishable in MS but exhibit intense low mass MS/MS signature ions that support quantitation.

To our knowledge there have been no studies to date that have used a proteomic approach to study the effects of warm ischemia on protein expression in Kupffer cells. We hypothe-

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The abbreviations used are: iTRAQ, isobaric tags for relative and absolute quantitation; AIF-1, allograft inflammatory factor-1; C3, complement factor 3; C3a, activated C3; FDR, false discovery rate; IL, interleukin.

sized that a proteomic approach might identify patterns of proteins that contribute to a more complete understanding of hepatic injury.

EXPERIMENTAL PROCEDURES

Animal Model—All animal experiments were carried out at the University of California at San Francisco with approval by the University of California at San Francisco Committee on Animal Research. Animal care was in agreement with the National Institutes of Health guidelines (57). Inbred male lean Zucker rats (Harlan Sprague-Dawley) were used for this study.

Lean Zucker rats ($n = 6$ each group) were randomized to either 75 min of warm ischemia or sham operation. Animals had access to standard laboratory diet and were maintained on a light-dark cycle. After anesthesia induction with isoflurane the liver was exposed through a midline incision. During the procedure the rats were actively warmed with heat pads and heat lamps to maintain a body temperature of greater than 37 °C as determined by continuous rectal temperature monitoring. Approximately 15 min passed between the induction of anesthesia and the onset of ischemia. We selected a 70% liver ischemia model because this is a well established model with reproducible results. In detail, vascular structures to the left and median lobe were identified and clamped for 75 min using a bulldog clamp. The unoccluded right and caudate lobe allowed outflow from the splanchnic circulation avoiding venous congestion. To confirm the appropriate placement of the clamps, the left and median lobes were inspected for signs of ischemia. During ischemia the abdomen was lightly packed with moist sponges, and the incision was approximated with clamps. After 75 min of warm ischemia the clamps were removed to allow reperfusion for 8 h. Animals received ~5 ml of normal saline intraperitoneally before closure of the incision. The rats in the sham group underwent the same surgical protocol with the exception that ischemia was not induced to the liver.

After 5 h of reperfusion, Kupffer cells were isolated according to Maher (15). Briefly Kupffer cells were isolated by Pronase and collagenase perfusion and digestion of the liver followed by Accudenz density gradient centrifugation.

Sample Preparation—The six samples from ischemia-treated and six from sham-operated animals were evaluated in six independent iTRAQ comparisons and cation exchange chromatography runs. For the comparisons, an equal amount of protein from isolated Kupffer cells harvested from the control animals was used. The protein concentration was measured after appropriate dilution with 20% Bio-Rad protein assay reagent (Bio-Rad) and 80% water according to Bradford (16). A Beckman photometer at 595 nm calibrated against air was used for all protein concentration measurements.

iTRAQ Labeling—iTRAQ labeling was performed using commercially available reagents (Applied Biosystems, Framingham, MA) based on the method described by Ross *et al.* (14).

Samples were first purified by methanol/chloroform precipitation as described previously (17). 20 μl of 100 mM triethylammonium bicarbonate (dissolution buffer) (Applied Biosystems) and 1 μl of 0.05% (w/v) SDS solution were added to each of the sample tubes containing 125 μg of protein. The tubes were vortexed, 2 mM tris(2-carboxyethyl)phosphine (reducing reagent; Applied Biosystems) were added, and the tubes were incubated at 60 °C for 1 h. The tubes were spun, then 1 μl of cysteine blocking reagent (Applied Biosystems) was added, and the tubes were incubated for 10 min at room temperature. Tryptic digestion was initiated by the addition of 2% (w/w) side chain-modified L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated porcine trypsin in double distilled H_2O. The reaction was allowed to proceed at 37 °C for 12 h. The tubes were spun, the iTRAQ reagent (Applied Biosystems) dissolved in 70 μl of ethanol was added to the tube, and the samples were incubated for 1 h at room temperature. The reaction was then quenched with 30 μl of double distilled H_2O. Labeling was checked by running an aliquot of the samples on the ESI-qQ-TOF mass spectrometer (see below) after purification with reverse phase C_{18} ZipTips (Millipore Corp.). A 5% fraction of the pooled samples was then run in one initial reverse phase and mass spectrometry run. The overall intensity of the stable isotope peaks were then compared to check for systematic errors in labeling and protein concentrations. The differentially labeled ischemia-treated and control samples were then pooled.

Each of the six sample pairs was then fractionated into 16 different fractions by cation exchange chromatography. Cation exchange chromatography was performed using an Amersham Biosciences Δkta system (Amersham Biosciences; 35-nl UV/visible cell, 1000-μl injection loop). A 2.0 × 10-mm polysulfoethyl A column with a 200 μl/min flow rate was used. For solvent A, 25% acetonitrile, 0.075% formic acid was used, and for solvent B, solvent A with additional 400 mM ammonium chloride was used. A gradient of 0% B, 0–10 min, 0–50% B, 3–60 min was set, and 16 fractions were collected from the mixture. Then the samples were brought to complete dryness in the SpeedVac and resuspended in 0.1% (v/v) formic acid. The fractions were desalted in an automated fashion using the Δkta system with a Jupiter C_{18} column.

Mass Spectrometry Measurements—Eight of the 16 fractions from each of the six labeled sample pairs were further separated by reverse phase chromatography. The separation of the peptides was achieved by a gradient of increasing acetonitrile in water (2–40%) over 60 min using 0.1% (v/v) formic acid as the ion pairing agent on a 75-μm inner diameter Jupiter Proteo reverse phase column. Tryptic peptides were subjected to LC-MS/MS analysis on a QSTAR electrospray mass spectrometer (Applied Biosystems) operating in positive ion mode that was connected in line with the chromatography unit as described elsewhere (18). Samples were separated by nano-liquid chromatography using a flow rate of 300 nl/min. Subsequently the sample was rerun three times with an exclusion list built from the peptides identified in the previous runs with all other settings being the same. A total of four mass spectrometry runs were performed for each fraction.

ELISA Measurements—To confirm the protein identification and quantitative results that were obtained by mass spectrometry, the protein content of myeloperoxidase and heat shock protein 70 were also measured by ELISA. Commercially available ELISA kits for these proteins were used (Calbiochem myeloperoxidase ELISA kit, catalog number 475918, EMD Biosciences, La Jolla, CA and HSP 70 ELISA kit, product number EKS-700, Stressgen Bioreagents, Victoria, British Columbia, Canada). Both kits have been shown previously to detect rat proteins (19) and are routinely used in our laboratory. 2 × 100 μg of each of the six samples from ischemia-treated rats and six sham-operated rats were used for both assays. Measurements were performed in duplicates according to the instructions of the manufacturer. The standard and control reagents that were provided with the kits were used. Results were calculated using the Dynex Revelation 4.22 ELISA reading software package. For comparisons with the iTRAQ results, for each of the six ischemia-sham sample pairs a ratio was calculated.

Data Analysis—The resulting 32 peak lists for each iTRAQ experiment (8 fractions × 4 runs each) were combined and interrogated against the Swiss-Prot rodent protein database using the ProteinProspector 4.13 software package (University of California at San Francisco, prospector.ucsf.edu (20, 21)). A minimal signal to noise threshold of five counts, a minimal ProteinProspector protein score of 15 and peptide score of 10, and a minimal discriminant score threshold of 1.0 were used for initial identification criteria. Peptides were first searched for matches to rat proteins; the peptides that had not matched were then searched against mouse protein entries. Protein

TABLE I
Oxidative proteins

The quantitation of 17 proteins associated with the cellular oxidant-antioxidant system met the criteria defined under "Experimental Procedures." The table shows the protein accession number, the name, the number of sample pairs in which the protein was quantified (Quant pairs), the number of significant peptides (Quant no. peptides), the mean iTRAQ ratio with standard deviation, and the *p* value if predefined criteria for statistical testing were met.

Swiss-Prot accession no.	Protein name	Quant pairs	Quant no. peptides	Mean ratio	S.D.	*p*
P04762	Catalase	6	80	1.23	0.42	0.0000
P07687	Epoxide hydrolase 1 (microsomal epoxide hydrolase)	4	23	0.97	0.10	0.1944
P48774	Glutathione *S*-transferase Mu 5 (GST class Mu 5)	2	17	1.72	0.52	0.0000
O35660	Glutathione *S*-transferase Mu 6 (GST class Mu 6)	4	25	1.20	0.45	0.0333
P00502	Glutathione *S*-transferase Ya-1 (ligandin)	6	73	1.34	0.33	0.0000
P04905	Glutathione *S*-transferase Yb-1 (chain 3)	4	25	0.79	0.26	0.0005
P08010	Glutathione *S*-transferase Yb-2 (chain 4)	5	32	1.37	0.47	0.0001
P08009	Glutathione *S*-transferase Yb-3 (chain 4)	6	51	1.11	0.32	0.0180
P04904	Glutathione *S*-transferase Yc-1 (chain 2)	6	45	1.33	0.39	0.0000
P11247	Myeloperoxidase precursor (MPO)	2	13	1.30	0.32	
Q63716	Peroxiredoxin 1 (thioredoxin peroxidase 2)	4	46	1.24	0.31	0.0000
O08807	Peroxiredoxin 4 (PRX-IV)	2	12	1.03	0.13	
Q9R063	Peroxiredoxin 5, mitochondrial precursor (PRX-V)	2	15	1.04	0.05	
O08709	Peroxiredoxin 6 (antioxidant protein 2)	6	59	1.32	0.32	0.0000
P07632	Cu,Zn-Superoxide dismutase	4	37	1.34	0.19	0.0000
P07895	Mn-Superoxide dismutase, mitochondrial precursor	3	18	1.20	0.39	0.0461
P50137	Transketolase (TK)	5	89	1.50	0.48	0.0000

identification and quantification for the proteins reported here was cross-checked manually. Quantification was determined by calculating the ratio of the areas under the reporter peaks (114.1 and 117.1) using the SearchCompare tool from the ProteinProspector suite of programs. Thresholds of quantitation on the basis of more than four significant peptide matches and their presence in more than two sample pairs were used. For each identified protein the mean and standard deviations of the peak intensity ratios from all peptides assigned to a given protein were calculated. Normal distribution was tested using the Kolmogorov-Smirnov test in SPSS 12.0 for Windows (SPSS Inc., Chicago, IL).

Statistical tests were only performed if the quantitative information was obtained from a minimum of eight peptides per comparison and in a minimum of two samples. One-sample *t* tests were used to calculate the *p* values for the rejection of the null hypothesis that the mean ratio of the peak areas is 1.0 (signifying no difference in the areas under the peptide peaks). Correction for multiple comparisons was done according to the false discovery rate method described by Benjamini *et al.* (22) using the FDRalgo software for Microsoft Windows made available to the public at www.math.tau.ac.il/~ybenja/ with a significance value of 0.05. *p* values larger than the FDR α value that was calculated using this software were considered statistically non-significant.

RESULTS

Interpreting the results of this study, we attempted to minimize potential bias by using one peptide for the identification the protein with the best match and ignoring less significant matches. Furthermore only quantitative results obtained from at least four independent peptides per sample and present in at least two sample pairs were used for quantitation. However, changes in the content of one relatively abundant protein might still influence ratios obtained for other, less abundant proteins that contain the same peptide.

Up-regulations led to statistically significant results in our study. Samples were normalized based on the total protein content, and proteins were quantified if they were present in both samples and controls. The cause for this phenomenon is most likely a bias due to a higher number of identified peptides per up-regulated protein in the ischemia group, leading to a lower number of quantitations for less abundant proteins.

Mass Spectrometry Measurements—Quantitative information was obtained for 1559 proteins. 185 of these proteins met the threshold criteria for quantitation (presence in at least two sample pairs with quantitation based on at least four peptides in each). On average the quantified proteins were present in 3.48 $\pm$ 1.51 sample pairs and were identified on the base of 42.38 $\pm$ 39.39 peptides.

Statistical comparisons from quantitative information obtained from more than 16 peptides in two sample pairs could be performed for 161 proteins with between 47 and 102 proteins in each of the six sample-control pairs. Testing for multiple comparisons by FDR analysis resulted in an FDR threshold (α) value of 0.0385886. After correction for multiple comparisons, statistically significant differences in two or more sample pairs were found in 20 proteins.

Oxidant and Antioxidant Proteins—Quantitative information for seven isoenzymes of the glutathione *S*-transferase was obtained. With the exception of the glutathione *S*-transferase Yb-1 (chain 3) all of these were present in statistically significantly increased concentrations in the Kupffer cells (Table I). Catalase was present in a 23% increased concentration after ischemia ($p < 0.0001$) (Table I and Fig. 1). The quantitation of four different peroxiredoxins showed an essentially unchanged cellular content in peroxiredoxins 4 and 5 (insuffi-

cient number of peptides for statistical testing) and an increase of 24 and 32% for peroxiredoxins 1 and 6 that was statistically significant ($p < 0.0001$). There was a 30% increase in the concentration of myeloperoxidase after ischemia (insufficient number of peptides for statistical testing) (Table I and Fig. 1). Two different isoforms of superoxide dismutase were quantified (Table I and Fig. 1). Content in Cu,Zn-superoxide dismutase was increased by 34% ($p < 0.0001$). The mitochondrial Mn-superoxide dismutase showed a similar trend with an overall increase of 20% ($p = 0.05$, did not meet FDR threshold).

Immunity- and Inflammation-related Proteins—There was an elevation in content of complement C3 precursor in Kupffer cells of 81% after warm ischemia ($p < 0.0001$; Table II and Fig. 2). On average the content of calgranulin B (S100A9) was elevated 81% after ischemia (Table II and Fig. 2). This elevation was statistically significant ($p = 0.0001$). The allograft inflammatory factor-1 (*AIF-1*) was elevated by 29% ($p < 0.01$) (Table II and Fig. 2).

Proteins of the Arachidonic Acid Metabolism—Three of the proteins associated with the arachidonic acid metabolism could be quantified (Table III and Fig. 3). The cellular content of the leukotriene A_4 hydrolase was elevated by 51% ($p < 0.0001$). Cyclooxygenase-1 was increased by 37% ($p = 0.0001$). There was only a 21% increase observed for the glutathione-requiring prostaglandin D synthase ($p = 0.01$).

ELISA Control Measurements—The myeloperoxidase standard curve resulted in a curve fit with an R^2 of 0.99. The ELISA detected myeloperoxidase in three of six sample pairs with an average content of 2.18 ± 3.32 ng/ml. The average ratio of the contents in ischemia/sham-operated animals was 1.13 ± 1.77. Using mass spectrometry, myeloperoxidase was quantified in two sample pairs, resulting in an average content ratio of 1.30 ± 0.33. Only in one sample pair was myeloperoxidase quantified by both methods; the content ratio for the ELISA measurement was 1.12 compared with 1.07 for the iTRAQ quantitation.

For heat shock protein 70 the standard curve resulted in a curve fit with an R^2 of 0.97. By ELISA heat shock protein 70 was quantified in five of six sample pairs with an average content of 22.35 ± 5.63 ng/ml. The average ratio of the contents in ischemia/sham-operated animals was 3.19 ± 2.02. Using mass spectrometry, heat shock protein 70 was quantified in the same five sample pairs, resulting in an average content ratio of 1.56 ± 0.61.

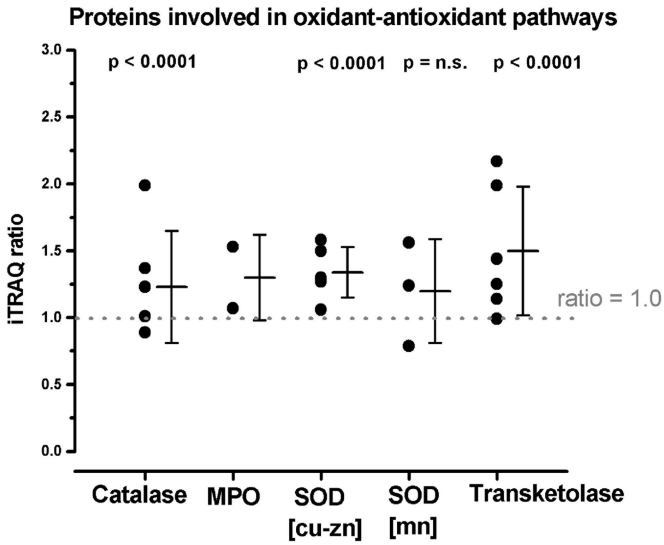

FIG. 1. **Oxidative proteins.** The proteins catalase, myeloperoxidase (*MPO*), different isoforms of the superoxide dismutase (*SOD*), and transketolase were elevated in Kupffer cells after liver ischemia. The myeloperoxidase quantitation did not meet the criteria for statistical analysis.

DISCUSSION

The present study demonstrated that the iTRAQ-based quantitative proteomic approach is able to monitor several different proteins under physiological and pathophysiological conditions. Several significant differences between the protein expression in rats undergoing hepatic ischemia and sham controls were observed. Our studies confirmed previously reported changes in the oxidative pathway. Importantly, however, iTRAQ-based quantitative proteomics demonstrated striking differences in several inflammatory proteins (*e.g.* calgranulin and allograft inflammatory factor) that have not been reported in the context of ischemia/reperfusion. Although some were predictable, others were novel, demonstrating the power of the iTRAQ method as a discovery tool.

Moreover the changes we found by iTRAQ comparison showed a trend very similar to the ones observed by ELISA in two proteins. This is noteworthy because a comparison of these methods of measurement could be biased by large number of factors, most importantly the recognition of protein fragments by ELISA and peptides by mass spectrometry that

TABLE II
Inflammatory proteins

The quantitation of three proteins associated with the cellular oxidant-antioxidant system met the criteria defined under "Experimental Procedures" (see legend for Table I for explanation of columns).

Swiss-Prot accession no.	Protein name	Quant pairs	Quant no. peptides	Mean ratio	S.D.	*p*
P55009	AIF-1	2	21	1.29	0.43	0.0063
P31725	Calgranulin B (migration inhibitory factor-related protein 14)	5	40	1.81	0.97	0.0000
P01026	Complement C3 precursor (contains C3a anaphylatoxin)	6	140	1.81	0.49	0.0000

are not recognized or assigned to a different protein by the other method. Sample size and the stringent statistical approach we used for mass spectrometry quantitation, however, were sufficient to expose the common trend with both methods.

The pathway for oxidant generation by neutrophils, monocytes, and macrophages begins with a membrane-associated NADPH oxidase that produces superoxide, which then dismutates to hydrogen peroxide (H_2O_2). Neutrophils and monocytes also secrete a heme protein, myeloperoxidase, which uses the oxidizing potential of H_2O_2 to convert chloride ion into the highly reactive hypochlorous acid (23). These reactive oxygen species are not only an important means of host defense against pathogens but have been shown to give rise to extensive tissue damage if not counterbalanced by antioxidants (24).

Superoxide dismutase has a role in both oxidant production and antioxidant defense. The transformation of superoxide to H_2O_2 provides the substrate for further transformation to H_2O by catalase. Both of these reactions occur in the intra- and extracellular space (23). The two main intracellular forms of superoxide dismutase are Cu,Zn-superoxide dismutase, which resides in the cytoplasm, and Mn-superoxide dismutase, which is found in the mitochondria. The increase that was found in both enzymes supports the hypothesis of an activation of the oxidant-antioxidant system in these cells.

Myeloperoxidase transforms hydrogen peroxide into several potentially damaging reactants (23). This enzyme is found in circulating neutrophils and monocytes but was only detected recently in Kupffer cells (25). Immunostaining with an antibody to proteins modified by hypochlorous acid, a characteristic product of the enzyme, indicated that myeloperoxidase is enzymatically active in cases of acute liver injury and cirrhosis (25). The increased cellular content of myeloperoxidase that we found supports the hypothesis that reactive oxygen species produced by Kupffer cells play an active role in the development of liver damage.

Glutathione *S*-transferase catalyzes the reaction of glutathione conjugation with electrophiles. The isoenzymes have been shown to be differentially expressed in different liver cells (26). The enzymes were found to be activated by ischemia/reperfusion in rats, resulting in a 1.8-fold increased activity when compared with the liver of untreated animals (27). These results correlate well with the increased cellular content that we found and are suggestive of an up-regulation of oxidant activity in Kupffer cells after ischemic liver injury.

Peroxiredoxins are a family of antioxidative proteins that currently has six members. They share a common reactive Cys residue in the N-terminal region and are capable of serving as a peroxidase and involve thioredoxin and/or glutathione as the electron donor. These proteins are distributed in the cytosol, mitochondria, peroxisome, and plasma. In addition to their role as a peroxidase, individual members most likely serve divergent functions that are associated with various biological processes such as the detoxification of oxidants, cell proliferation, differentiation, and gene expression (28). Peroxiredoxins can be regulated by changes to phosphorylation, redox, and possibly oligomerization. They share the same basic catalytic mechanism in which an active site cysteine is oxidized to a sulfenic acid by the peroxide substrate (29, 30). Intracellular peroxiredoxins might act as a dam against oxidative stress, and the ratio of active to inactive enzyme might play a role in whether cells are susceptible to cytokine-induced apoptosis (31). The increased cellular con-

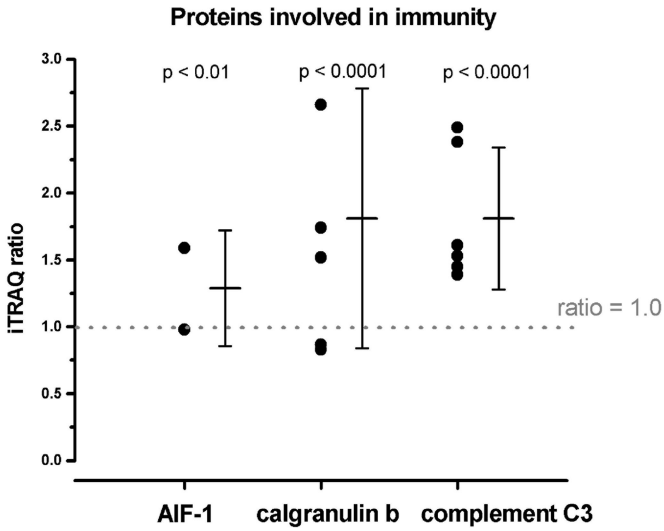

FIG. 2. **Immunity-related proteins.** The *data points* represent the average peptide ratios in each of the ischemia-control pairs; the *error bars* on their *right* represent the overall means and standard deviations. The three immunity-related proteins AIF-1, calgranulin B, and complement C3 were significantly elevated after ischemia.

TABLE III
Proteins of the arachidonic acid metabolism

Quantitative information was sufficient for three proteins associated with the arachidonic acid metabolism (see legend for Table I for explanation of columns).

Swiss-Prot accession no.	Protein name	Quant pairs	Quant no. peptides	Mean ratio	S.D.	*p*
P30349	Leukotriene A_4 hydrolase (LTA_4 hydrolase)	6	72	1.51	0.46	0.0000
P22437	Prostaglandin G/H synthase 1 precursor (cyclooxygenase-1)	4	35	1.37	0.37	0.0000
Q9JHF7	Glutathione-requiring prostaglandin D synthase (glutathione-dependent PGD synthetase)	5	41	1.21	0.53	0.0140

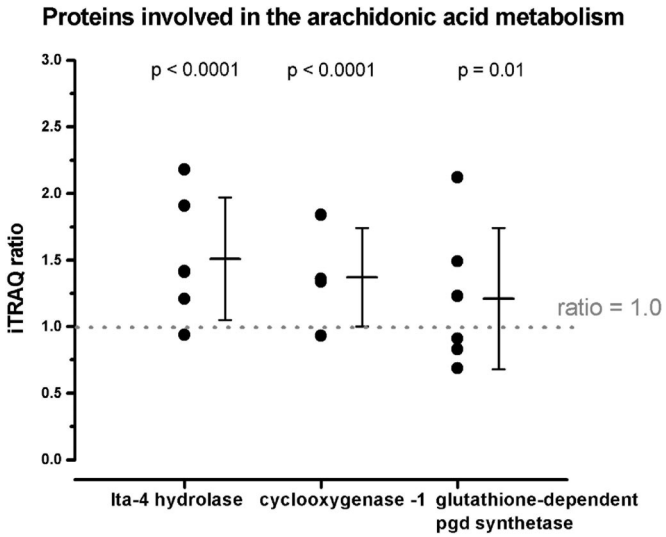

FIG. 3. **Proteins of the arachidonic acid metabolism.** There was significantly increased cellular content in leukotriene A_4 (*lta-4*) hydrolase, prostaglandin G/H synthase 1 precursor (*cyclooxygenase-1*), and glutathione-requiring prostaglandin D synthase (*glutathione-dependent pgd synthetase*) in Kupffer cells after liver ischemia.

tent of peroxiredoxins 1 and 6 that we found is in agreement with recent studies that showed pI changes in peroxiredoxins 1, 2, and 6 after cell treatment with oxidants, most likely as a result of oxidative changes (31, 32), probably indicating an increased turnover of these proteins.

Immunity- and Inflammation-related Proteins—Complement factor 3 (C3) is the fourth component to attach in the complement reaction sequence. It is a β-globulin with a molecular mass of 185 kDa. Its fragments affect smooth muscle and have anaphylatoxic, chemotactic, and histaminic action. Possible sources of C3 in Kupffer cells include production by the cell itself and uptake of C3 from the extracellular space (33, 34). The increased cellular content of C3 that we found is in agreement with a previous investigation describing an increase in C3 release by Kupffer cells after lipopolysaccharide treatment of guinea pigs (34). It has further been shown that Kupffer cell activation by xenotransfusion leads to an increased uptake of plasma C3 in the cytoplasm of these cells (35), a finding that is supported by the expression of CR1, CR3, and CR4 complement receptors on Kupffer cells (36). Light and electron microscopic immunocytochemistry of liver biopsy specimens from human patients with and without liver disease found that C3 was not in the protein-synthesizing apparatus but was in the phagosomes of Kupffer cells (37); the percentage of internally produced C3 of the overall C3 present in Kupffer cells might therefore be small. However, increased production may be a factor in the increased cellular content of C3 that we found.

Further Interesting Findings Involve the AIF-1 and Calgranulin B (S100A9)—AIF-1 is a modulator of the immune response during macrophage activation whose expression is up-regulated by interferon-γ (38). In a macrophage cell line, AIF-1 has been reported to increase production of IL-6, IL-10, and IL-12p40 but not IL-12p70 compared with control cells. No difference was noted in production of tumor necrosis factor-α, transforming growth factor-β1, and IL-1α (39). Increased numbers of AIF-1-immunoreactive macrophages have been observed in brain infarctions and injury, gliomas, rat uveitis, and inflammatory lesions of a rat model of autoimmune disease (38). Furthermore *AIF-1* transcript levels have been shown to be increased in rejection of the heart and kidney (40, 41).

Similarly Nagakawa *et al.* (42) demonstrated recently that *AIF-1* is overexpressed in liver allografts with acute rejection after transplantation in rats. However, AIF-1 was only slightly increased in syngenic liver transplantation (hence cold ischemia/reperfusion model) at day 3. In contrast, we found already significantly elevated *AIF-1* expression after 5 h of reperfusion in a warm ischemia model. Elevated *AIF-1* content has never been reported in a warm ischemia/reperfusion model.

Therapy with high doses of recombinant autoantigens or dexamethasone has lead to a reduction of AIF-1-immunoreactive macrophages (43). In addition, *AIF-1* levels were significantly decreased in allografted animals receiving immunosuppression (44). Interestingly animals treated with immunosuppression before ischemia/reperfusion showed ameliorated organ injury (45–47). The increased concentrations that we found are an indicator of the up-regulation of Kupffer cells after warm ischemic liver injury and a potential involvement of interferon-γ-mediated protein cascades and provide further evidence that ischemia/reperfusion injury may contribute to allograft rejection.

Of even greater magnitude was the striking overexpression (89%) of calgranulin B (S100A9), which in conjunction with calgranulin A (S100A8) is a prominent player in innate immunity (48). Calgranulin B (molecular mass, 13.2 kDa) is a calcium-binding protein that is known to form complexes with calgranulin A and other proteins. Calgranulin A (S100A8) was identified in our samples as well, but the number of peptides was below the quantitation threshold (the iTRAQ ratio from 15 peptides originating from four experiments was 3.57 $\pm$ 2.14). Calgranulin B is expressed at high concentrations in granulocytes as well (49). The contribution of the innate immunity to ischemia/reperfusion injury is increasingly recognized and investigated. As in the case of AIF-1, acute allograft rejection revealed expression of calgranulins A and B. The calgranulin complex has also been shown to induce apoptosis in colon cancer cell lines (50). Apoptosis is a well known feature of cell death during ischemia/reperfusion.

The significantly increased cellular content that we found is an interesting starting point for further investigations because calgranulin B is known to have a number of important roles in intracellular pathways (51). For example, calgranulin B has been associated with T-cell responses and affinity changes of the Mac-1 integrin (52).

Furthermore the calgranulin complex is closely linked to the arachidonic acid metabolism in human neutrophils and ap-

pears to be a mediator between calcium signaling and arachidonic acid effects (49). Previous work has demonstrated an increase in Kupffer cell prostaglandin E_2 production in response to sodium butyrate (53).

Our observations support the hypothesis that the C3a-dependent activation of hepatic glycogenolysis is mediated by way of a C3a-induced prostanoid production in Kupffer cells. There are experimental data that C3a dose-dependently increases prostaglandin D_2, thromboxane B_2, and prostaglandin $F_2\alpha$ formation in Kupffer cells (54). In another study, Kupffer cells from lipopolysaccharide-treated animals released significantly greater amounts of prostaglandin E_2 than control animals when stimulated *in vitro* with lipopolysaccharide, indicating a priming of activated Kupffer cells for prostaglandin E_2 production (34).

In summary, our results demonstrate that the application of quantitative proteomics to specifically isolated cells from an animal model is a valid approach to evaluate cellular changes with different stimuli. However, it is important to remember that that the concentration changes of the relatively abundant proteins we found is only one factor in the cellular response. Post-translational modifications are an important component of cellular reactions to stimuli that are unlikely to be detected by the approach used here and will need to be addressed in future studies (55, 56).

We were able to identify and quantify a large number of proteins from Kupffer cells. Our results indicate significant alterations of proteins that have important functional consequences in the intracellular oxidant-antioxidant system, the inflammatory response, and the arachidonic acid metabolism. In particular, we demonstrated for the first time that proteins involved in organ rejection are also overexpressed in a warm ischemia liver model. This underscores the importance and possible link of inflammation and innate immunity in both ischemia/reperfusion injury and organ rejection.

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