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Alterations in the proteome of pulmonary alveolar type II cells in the rat after hepatic ischemia-reperfusion

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Abstract

Objective—Hepatic ischemia-reperfusion can be associated with acute lung injury. Alveolar epithelial type II cells (ATII) play an important role in maintaining lung homeostasis in acute lung injury.

Design—To study potentially new mechanisms of hepatic ischemia-reperfusion-induced lung injury, we examined how liver ischemia-reperfusion altered the proteome of ATII.

Setting—Laboratory investigation.

Subjects—Spontaneously breathing male Zucker rats.

Interventions—Rats were anesthetized with isoflurane. The vascular supply to the left and medial lobe of the liver was clamped for 75 mins and then reperfused. Sham-operated rats were used as controls. After 8 hrs, rats were killed.

Measurements and Main Results—Bronchoalveolar lavage and differential cell counts were performed, and tumor necrosis factor-α and cytokine-induced neutrophil chemotactic factor-1 in plasma were determined by enzyme-linked immunosorbent assay. ATII were isolated, lysed, tryptically digested, and labeled using isobaric tags (iTRAQ). The samples were fractionated by cation exchange chromatography, separated by high-performance liquid-chromatography, and identified using electrospray tandem mass spectrometry. Spectra were interrogated and quantified using ProteinProspector. Quantitative proteomics provided quantitative data for 94 and 97 proteins in the two groups. Significant changes in ATII protein content included 30% to 40% increases in adenosine triphosphate synthases, adenosine triphosphate/adenosine diphosphate translocase, and catalase (all $p < .001$). Following liver ischemia-reperfusion, there was also a significant increase in the percentage of neutrophils in bronchoalveolar lavage (48% ± 26%) compared with sham-operated controls (5% ± 3%) ($p < .01$), and plasma tumor necrosis factor-α levels were also significantly increased.

Conclusions—The proteins identified by quantitative proteomics indicated significant changes in moderators of cell metabolism and host defense in ATII. These findings provide new insights into possible mechanisms responsible for hepatic ischemia-reperfusion-related acute lung injury and suggest that ATII cells in the lung sense and respond to hepatic injury.

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Keywords

liver; lung; quantitative proteomics; isotope ratio mass spectrometry; ischemia; reperfusion injury; Zucker rats; iTRAQ

Pulmonary edema has been observed within hours of liver transplantation (1,2), and in animal experiments pulmonary septal thickening and neutrophil influx in the lung have been observed after transient hepatic artery clamping with liver injury (3). This form of lung injury has been attributed to hepatic ischemia-reperfusion (I/R) injury (3-6), but the underlying mechanisms have not been fully elucidated. One proposed mechanism is that the activation of hepatic Kupffer cells that occurs during I/R results in the production of oxygen radicals and the modulation of proinflammatory and anti-inflammatory cytokines (7,8). It is generally assumed that the damage to the pulmonary epithelium is transmitted by mediators released from the liver into the bloodstream, since the lungs are the first capillary bed that is reached by the blood after leaving the hepatic circulation. Various cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, prostaglandins, and reactive oxygen species (3,5,9), have been shown to be released after liver I/R. The alveolar epithelium seems a likely target of such an injury, since it has been shown that brief (2 × 15 mins) clamping of hepatic inflow induces lung injury if combined with a high tidal volume ventilation regimen that was not injurious alone (10). We hypothesized an important role of the alveolar type II cell (ATII), since this cell type is well known to perform key functions in the normal lung and in lung injury and repair, including regulation of surfactant metabolism, ion and fluid transport, and alveolar repair (11).

Rapid progress in both protein separation and identification techniques has made mass spectrometry a valuable tool for characterizing *in vivo* proteins (12-18). One goal of proteome research is to evaluate the responses to different stimuli (19,20) and to discover alterations in pathways that can then be further evaluated (21,22). We have recently reported that a modern quantitative proteomic approach is capable of quantifying changes in protein profiles, namely of the reactive oxidant and prostaglandin producing systems, in hepatic Kupffer cells after liver injury (8) and ATII after mechanical ventilation (23).

Therefore, the primary objective of this study was to use a proteomics approach to develop testable hypotheses that may identify mechanisms of injury to the pulmonary epithelium following hepatic I/R (8,21,23).

EXPERIMENTAL PROCEDURES

Animal Model

All animal experiments were carried out at the University of California at San Francisco (UCSF) with approval by the UCSF Committee on Animal Research. Animal care was in agreement with the National Institutes of Health guidelines for ethical research (NIH publication 80-123, revised 1985). Inbred male lean Zucker rats (Harlan Sprague Dawley, Indianapolis, IN) were used for this study.

Lean Zucker rats (n = 4 in the I/R and sham-operated groups, n = 8 normal control animals, all animals between 250–300 g) were randomized to either 75 mins of warm I/R or sham operation. The rats 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. The procedure was performed by two experienced surgeons in sterile technique. During the procedure, the rats were actively warmed with heat pads and heat lamps to maintain a body temperature >37°C as determined by continuous rectal temperature monitoring. Approximately 15 mins passed between the induction of anesthesia and the onset of ischemia.

We selected a 70% liver ischemia model with reproducible hepatic injury (8). Vascular structures to the left and median lobe were identified and clamped for 75 mins using a vascular clamp. The 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 mins of warm ischemia, the clamps were removed to allow reperfusion for 8 hrs. The rats received approximately 5 mL of normal saline intraperitoneally before closure of the incision. The rats in the sham group underwent the same surgical protocol without hepatic ischemia.

Sample Preparation

After 8 hrs of reperfusion, the rats were killed by transsection of the abdominal aorta under isoflurane anesthesia. Bronchoalveolar lavage was done in all animals with 7 mL of normal saline. ATII were isolated as previously described (11,23). The cells in the supernatant were then collected by centrifugation, transferred to cell preservation medium (10% fetal calf serum, 10% dimethyl sulfoxide, in minimum essential medium, UCSF Cell Culture Facility), and stored at −80°C until further processing. An average of 76×10^6 type II cells could be obtained. Cell viability was monitored immediately after cell isolation by light microscopy in a hemocytometer after addition of tryptane blue. Cell purity was evaluated after thawing immediately before cell lysis by Wright-Giemsa staining and light microscopy in all samples (I/R, 93.3% ± 1.4%; sham, 93.8% ± 1.2%; normal controls, 92.7% ± 2.6%; p = not significant). Cells were normalized by cell count, but protein concentration was monitored in all samples (Table 1).

ELISA Measurements

Tumor necrosis factor-α and cytokine-induced neutrophil chemotactic factor (CINC-1) were measured in undiluted plasma using commercially available enzyme-linked immunosorbent assays (ELISA) (TNF-α, Pierce Biotechnology, Rockford, IL; CINC-1, R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer.

iTRAQ Labeling

Four samples from I/R-treated animals and four from sham-operated animals were evaluated in four independent comparisons using isobaric tags for relative and absolute quantitation (iTRAQ, Applied Biosystems, Framingham, MA) and cation exchange chromatography runs. iTRAQ labeling was performed using commercially available reagents based on the method described by Ross et al. (24) as previously described (8). Samples were first purified by methanol/chloroform precipitation as described previously (25). After labeling, the differentially labeled I/R-treated and control samples were pooled.

Each of the four sample pairs was then fractionated into 16 different fractions by cation exchange chromatography. Cation exchange chromatography was performed using an Amersham Biotechnology Äkta system (Amersham Biotechnology, Piscataway, NJ; 35-nL UV/V cell, 1000-μL injection loop) as previously described (8,23).

Mass Spectrometry Measurements

Eight of the 16 cation exchange fractions from each of the four labeled sample pairs were further separated by reverse phase chromatography as previously described (8,23). Tryptic peptides were subjected to liquid chromatography-tandem mass spectrometry 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 (26). Samples were separated by nano-liquid chromatography using a flow rate of 300 nL/min. Subsequently,

the sample was repeated three times with an exclusion list built from the peptides identified in the previous run with all other settings being the same. Four mass spectrometry runs were performed for each fraction.

Data Analysis

The results of the ELISA and bronchoalveolar lavage measurements were compared using the Student's *t*-test for unpaired samples in SPSS 12.0 for Windows (SPSS, Chicago, IL). Data are shown as mean ± SD.

The 32 peak lists for each iTRAQ experiment (eight fractions at four runs each) were combined and interrogated against the Swissprot rodent protein database using the ProteinProspector 4.13 software package (University of California, San Francisco, prospector. ucsf.edu) (27,28)). A minimal signal-to-noise threshold of five counts, a minimal protein prospector 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 identification and quantification for the proteins reported here were cross-checked manually. Quantification was determined by calculating the ratio of the areas under the reporter peaks using the SearchCompare tool from the ProteinProspector suite of programs. Thresholds of quantitation on the basis of at least five significant peptide matches 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.

To correct for any potential bias due to inconsistencies in labeling, treatment and control samples were labeled in an alternating fashion and the results were normalized to an average protein content ratio of 1.0 between I/R and sham-operated samples and normal controls (8, 23). One-sample Student's *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 (FDR) method described by Benjamini et al. (29) using the FDRalgo software for MS Windows with a significance value of .05. *P* values larger than the FDR α value that was calculated using this software were considered statistically nonsignificant.

To evaluate the combined interexperimental and interindividual variability, statistical comparison between the nonoperated control samples was performed for the proteins that had sufficient data for quantitation after liver I/R and sham operation. The *t*-tests, correction for multiple comparisons, and all other parameters were applied using the same thresholds as for the comparisons between treatment and control groups. Ideally, we expected <5% statistically significant differences with the significance level of .05 that was used throughout the experiments. For the proteins that met the thresholds for quantitation in the I/R group, five of 95 Student's *t*-tests were statistically significant (5.2%), whereas four of 80 (5%) tests were statistically significant for the proteins quantified the sham-operated groups.

RESULTS

Bronchoalveolar Lavage

There was a significant increase in the number of cells per milliliter of bronchoalveolar lavage after sham operation ($p < .05$) and liver I/R ($p < .01$) compared with normal controls. The cell differential resulted in a significant increase in the percentage of bronchoalveolar lavage neutrophils after I/R (48% ± 26%) compared with sham-operated controls (5% ± 3%) ($p < .01$) (Table 1).

ELISA Measurements

The TNF-α concentration in plasma of I/R-treated rats was 219 ± 180 pg/mL (n = 6) (Fig. 1*A*), whereas the cytokine had an average content of 7 ± 15 pg/mL in the plasma of normal controls (n = 5). TNF-α was not detected in plasma samples from sham-operated rats. The IL-8 analog CINC-1 was significantly elevated in the plasma of I/R-treated animals (1537 ± 279 pg/mL; n = 6) (Fig. 1*B*) compared with both normal controls (204 ± 128 pg/mL; $p < .0001$) and sham-operated rats (930 ± 140 pg/mL; $p < .001$). The difference in plasma CINC-1 content between normal controls and sham-operated rats was statistically significant as well ($p < .0001$).

Mass Spectrometry Measurements

Data were obtained for 1,513 proteins. Ninety-four of these proteins met the threshold criteria for quantitation and statistical comparison in the I/R-treated group and 97 in the sham-operated group. On average, the quantified proteins were identified on the base of 29 ± 6 peptides. Testing for multiple comparisons by FDR analysis resulted in an FDR threshold (α) value >.002 for the I/R-treated group and .001 for the sham-operated group. In the statistical comparison between the nonoperated control samples to evaluate variability for the proteins that met the thresholds for quantitation in the I/R group, five of 95 Student's *t*-tests were statistically significant (5.2%), whereas four of 80 (5%) tests were statistically significant for the proteins that were quantified in the sham-operated animals.

Oxidant Proteins

Catalase (Table 2 and Fig. 2) was present in a 34% increased concentration in the rats after I/R ($p < .0001$), whereas there was no significant difference in its content between sham-operated rats and normal controls. The difference in its content between the I/R and sham-operated groups was significant as well ($p < .0001$). There was a 52% increase in the cellular content of myeloperoxidase in rats after I/R ($p < .0001$). The enzyme content was elevated to a lesser degree, but the increase was statistically significant ($p < .0001$), in animals after sham operation. The difference between the I/R and the sham groups was also significant ($p < .0001$). Only one isoform of superoxide dismutase, [Cu-Zn], was quantified. Compared with controls, the content of superoxide dismutase [Cu-Zn] was not altered in the I/R or the sham-operated groups (*p* not significant), although there was a significant difference in the direct comparison of the two groups ($p < .0005$).

Adenosine Phosphate Generating Enzymes

All quantified enzymes of the adenosine triphosphate (ATP)-generating pathways followed the same pattern. In rats after both liver I/R and sham operation, a similar and statistically significantly elevated content in the cellular content of adenosine diphosphate/ATP translocase 2 of 37% ($p = .0001$) and 28% ($p < .0001$) (Fig. 3 and Table 3) was found. An increased content in the β chain of the mitochondrial ATP synthase was observed in rats after liver I/R (31%, $p < .0001$) and sham operation (16%, $p < .0001$). The α chain of the mitochondrial ATP synthase was increased by 39% in the liver I/R group ($p < .001$) and by 24% after sham operation ($p < .0001$).

Cellular Enzymes

The protein content of the quantified cellular enzymes followed two distinct patterns: Nine of these enzymes were statistically significantly up-regulated in rats after liver I/R, whereas the cellular content in animals after sham operation was not significantly changed (Table 4 and Fig. 4). This group consisted of aldehyde dehydrogenase (43% increase with I/R, $p < .0001$), aspartate aminotransferase (42% increase, $p < .0001$), carboxylesterase 3 (42% increase, $p < .0001$), fatty acid synthase (39% increase, $p < .0001$), L-lactate dehydrogenase (49% increase,

$p < .0001$), mitochondrial malate dehydrogenase (43% increase, $p < .0001$), and three isoforms of the protein disulfide isomerase (A3, 64% increase, $p < .0001$; A6, 43% increase, $p < .0005$; and precursor, 32% increase, $p < .0001$). The cellular content of the remaining enzymes did not change statistically significantly after any of the interventions.

Regulatory Proteins

Quantitative information for several cell regulatory proteins (Table 5) was obtained. The cellular content changes that were observed in these regulatory proteins in rats with and without liver injury can be subdivided into three different patterns. The contents of a first group were statistically significantly increased in the group after liver I/R but not in the group after sham operation. These were 78-kDA steroidogenesis activator polypeptide (46% increase, $p < .0005$), calregulin (35% increase, $p < .0001$), elongation factor 1-α1 (25% increase, $p < .001$), mitochondrial 10-kDA heat shock protein (Hsp) (55% increase, $p < .003$), Hsp 60 (54% increase, $p < .002$), annexin A5 (36% increase, $p < .0001$), protein kinase C inhibitor protein 1 (50% increase, $p < .001$), and tumor rejection antigen gp 96 (28 increase, $p < .0001$). The cellular contents in a second group of proteins were significantly increased in both the liver I/R and the sham operation groups. Members of this group were members of the annexin family (A1, 46% increase in animals after I/R, $p < .0001$, and 23% increase in rats after sham operation, $p < .001$; A6, I/R +35%, $p < .0001$ and sham +16%, $p < .0001$). The cellular content of the remaining proteins was not statistically significantly altered in rats after any intervention, although some of the remaining proteins seemed to have a noticeable but not statistically significant trend toward an increased cellular content in rats after liver I/R. These proteins included annexin A2 (I/R +33%, $p < .02$, and sham +31%, $p = .001$) as well as calcyclin, heat shock cognate 71-kDa protein, Hsp 90 β, lymphocyte cytosolic protein 1, prohibitin, and ubiquitin.

Structural Proteins

Measured differences in the cellular content in structural proteins (Table 6) could be subdivided into two different patterns. The first group of proteins was significantly increased in the group after liver I/R but not in the group after sham operation. These proteins were β-actin (26% increase, $p < .0005$), cytokeratin 8 (52% increase, $p < .0001$), lamin A (22% increase, $p < .0001$), lamin B1 (50% increase, $p < .0001$), moesin (20% increase, $p < .001$), α-spectrin (44% increase, $p < .0005$), β spectrin (45% increase, $p < .0001$), and vimentin (44% increase, $p < .0001$). The cellular content of other proteins was significantly increased in the animals that had undergone either liver I/R or sham operation. Members of this group were myosins (M-14: I/R +47%, $p < .0001$; sham +22%, $p < .0001$; M-9: I/R 347%, $p < .0001$, sham +20%, $p < .0001$) and plectin-1 (I/R +43%, $p < .0001$; sham +30%, $p < .0001$).

DISCUSSION

Several of the observed changes in the proteome of alveolar epithelial type II cells suggest potential new pathologic mechanisms of how short-term hepatic I/R may initiate injury to the pulmonary epithelium. Compared with previous data in a porcine model (6), our model is characterized by a more pronounced bronchoalveolar lavage neutrophilia. We also determined that hepatic I/R induced a significant increase in plasma levels of proinflammatory cytokines (TNF-α, CINC-1). The parallel course of plasma TNF-α and CINC-1 increase and bronchoalveolar lavage neutrophilia is not unexpected, given the likely role of TNF-α and CXC cytokines, such as CINC-1, as chemoattractors for polymorphonuclear cells (30). The CINC-1 increase after sham operation makes this cytokine a potentially less specific plasma marker in this model.

Proteins of the Oxidant Syste

Proteome analysis demonstrated an up-regulation of the oxidative pathway in ATII cells following hepatic I/R. In neutrophils and monocytes, myeloperoxidase participates in the generation of reactive oxygen species, an important mechanism for host defense against pathogens. Previous studies have shown that ATII express myeloperoxidase; its function in these cells is uncertain (31). Conversely, catalase and superoxide dismutase have primarily an antioxidant function: Superoxide dismutase catalyzes the transformation of superoxide to H_2O_2, providing the substrate for further transformation to H_2O by catalase. Both of these reactions occur in the intra- and extracellular spaces (32). Treatment with N-acetyl-1-cysteine, an oxidant scavenger, has been shown to prevent lung injury (33).

Enzymes of the Adenosine Phosphate System

Enzymes of the adenosine phosphate pathway in ATII were significantly increased in the liver I/R group and to a lesser degree in the sham-operated group. ATP synthase activity has been associated with the content of ATP synthase β in the inner mitochondrial membrane (34). However, regulation of cellular ATP synthase is dynamic, and β-chain expression is not always associated with ATP synthase activity (34). Our findings indicate that the cellular protein content of these enzymes could be a sensitive marker for changes in cell metabolic activity.

Cellular Enzymes

Following liver I/R, the intracellular levels of several enzymes in ATII cells were significantly elevated. Aldehyde dehydrogenase 1 catalyzes the pyridine nucleotide-dependent oxidation of aldehydes to acids and plays an important role in detoxification. This may increase lung vulnerability, since the lung is well known to be a major target for toxicity from inhaled aldehydes (35). Carboxylesterase 3 messenger RNA has previously been localized in the airway epithelium (36), and the cellular content of this enzyme has been demonstrated to be increased after I/R injury of the rat lung (37); it may serve to detoxify inhaled organophosphorus agents (36). Protein disulfide isomerase catalyzes oxidative protein folding and has been shown to introduce disulfide bonds into folding proteins (38). The cellular content of two ubiquitous enzymes involved in amino acid metabolism, mitochondrial aspartate aminotransferase and L-lactate dehydrogenase, was elevated to a similar degree, which may suggest increased protein synthesis. Malate dehydrogenase is an enzyme in the citric acid cycle that catalyzes the conversion of malate into oxaloacetate using nicotinamide adenine dinucleotide. Regulating factors in liver cells include T3, glucagon, insulin, glucocorticoids, and fatty acids (39). Fatty acid synthase is an enzyme involved in fatty acid synthesis from glucose. It is a key enzyme in the biosynthesis of lung surfactant. Its expression in fetal lungs is increased by glucocorticoids, and this effect is largely due to increased transcription (40).

Regulatory Proteins

Folding of polypeptides in the cell typically requires the assistance of several protein systems termed *molecular chaperones*. The Hsp 60 system, consisting of GroEL (Hsp 60) and its cofactor GroES (Hsp 10), assists in the protein folding to expose hydrophobic surfaces (41). Serum levels of Hsp 60 detected within 30 mins after trauma have been associated with the development of acute lung injury (42). Hsp 60 induces release of nitric oxide by macrophages, implicating extracellular release in the activation of the pulmonary inflammatory response (42). The increased intracellular content of both Hsp 60 and Hsp 10 in ATII cells that we observed after liver I/R is therefore a potential indicator of a stress response in the lung following liver injury. Similarly, 78-kDA steroidogenesis activator polypeptide, also referred to as GRP 78 or BiP, was increased in lung ATII after liver I/R. This protein is considered a central regulator and marker for endoplasmatic reticulum stress due to its role as a major endoplasmatic reticulum chaperone with anti-apoptotic properties (43). A related protein is

calregulin; it is part of an endoplasmatic reticulum chaperone system that ensures the proper folding and quality control of newly synthesized glycoproteins (44). Elongation factor 1-α is a core component of protein synthesis with a primary function in the process of kinetic proofreading that results in appropriate codon-anticodon binding interactions (45).

Annexins are a family of calcium- and phospholipid-binding proteins that appear to be involved in membrane fusion and signal transduction (31). The annexin A5 (lipocortin V) content in the ATII was significantly elevated after liver I/R but not after sham operation. A5 binds to phosphatidylserine, one of the apoptosis signals at the surface of an apoptotic cell, and it is widely used to detect apoptosis (46,47). An increased level of A5 could therefore suggest an increased rate of apoptosis. The cellular content in other members of the annexin family was significantly increased after both liver I/R and sham operation. Annexin A1 has a large number of functions, including inhibition of expression of inducible nitric oxide synthase in macrophages, shedding of L-selectin and binding to the $\alpha_{4\beta1}$-integrin, targeting cells for nonphlogistic destruction, regulation of neutrophil transmigration, and inhibition of phospholipase A_2 (48). Moreover, recent studies suggest that A2 plays a role in the cytoskeleton reorganization in stimulated type II cells, allowing surfactant-containing lamellar bodies to access the plasma membrane (32). The increase in protein kinase C inhibitor protein 1 might indicate an activity change in protein kinase C, which plays a ubiquitous role in intracellular signal transduction (49). Protein kinase C participates in cellular processes, including growth, differentiation, and apoptosis.

Structural Proteins

In addition to maintaining the structural integrity of cells, structural proteins have roles in cell signaling and microvesicle formation. Changes in the actin cytoskeleton are known to contribute to neutrophil migration from the vasculature in the lung epithelium (50) and most likely to cell repair (51). Lamins are intermediate filament proteins. Whereas B-type lamins are ubiquitously expressed in all animal cells, the expression of A-type lamins is low or absent in cells with a low degree of differentiation and/or in highly proliferating cells (37,38). Ezrin, radixin, moesin, and merlin form a subfamily and act as links between the plasma membrane and the cytoskeleton, interacting with each other and with cell adhesion molecules important in repair and fibrosis, such as CD44, and with F-actin (52). A recent proteomics study on lung fibroblasts in patients with lung fibrosis found increased concentrations of moesin (53). Spectrin is involved in secretion and interacts with calmodulin in a calcium-dependent manner. It plays a role in clustering and regulation of proteins associated with the plasma membrane, control of morphogenesis and cell proliferation, protein sorting and trafficking in the Golgi apparatus and in intracellular vesicles, architecture of the nucleoplasm, and regulation of transcription factors in the nucleus (33). Moreover, it has been demonstrated that the putative pore-forming subunit of the rat epithelial (amiloride-sensitive) Na^+ channel (α ENaC) binds to α-spectrin *in vivo* (34).

Outlook and Limitations

The most important limitation of our study is that this liver ischemia and reperfusion model does not represent all types of hepatic ischemia. It is well established as a model of hepatic surgery with a transient interruption of perfusion to the whole organ REF, but it cannot provide information for prolonged states of low flow, as they may occur during periods of low cardiac output or prolonged shock, or focal ischemia. Further studies are needed to evaluate the impact of these conditions on the lung.

This experimental study shows alterations in the cellular content of enzymes, regulatory proteins, and structural proteins that may indicate potential pathways of secondary injury to the lung following liver I/R. Quantitative proteomics of isolated epithelial cells proved to be

a promising discovery tool to evaluate early cellular changes in response to injury in one organ (the lung) that is anatomically separated from the causative organ (the liver). Such a systemic stimulus to the alveolar epithelium could be transmitted by mediators released from the liver into the bloodstream because the lungs are the first capillary bed perfused by the blood leaving the hepatic circulation. Numerous cytokines, including TNF-α, IL-1β, prostaglandins, and reactive oxygen species (5,9,10), have been implicated in the pathogenesis of secondary pulmonary injury, and previous studies have shown that hepatic Kupffer cells play an important role in the production of these mediators following hepatic parenchymal cell injury (7,8). However, the specific signaling mechanisms for ATII cell injury after liver I/R remain to be elucidated.

Interestingly, we found mainly up-regulation of proteins after this type of indirect lung injury. In contrast, in our previous study of direct injury to the pulmonary epithelium by ventilator-induced injury (23), the protein content in key proteins (e.g., the proteins of the adenosine phosphate pathway) was decreased. This result may suggest that damage to the ATII cell plays a role in ventilator-induced lung injury, whereas the secondary injury due to liver I/R may lead primarily to an activation of the ATII.

The change in the concentration of relatively abundant proteins is only one factor in the cellular response to injury. A large part of the cellular response to injury includes posttranslational modifications of existing proteins that result in little or no change in cellular protein content and will need to be addressed in future studies (21,54).

SUMMARY

The results of this experimental study suggest that hepatic I/R induces significant alterations in the expression of proteins in alveolar epithelial type II cells in the lung. The identified proteins have important functions in the intracellular oxidant-antioxidant system, the adenosine phosphate system, several cellular enzymes cascades important in the response to stress, regulation of apoptosis, and cell structure. These alterations in key proteins may represent the earliest biochemical events associated with the development of lung injury secondary to hepatic I/R. Further studies are needed to test the role of these pathways.

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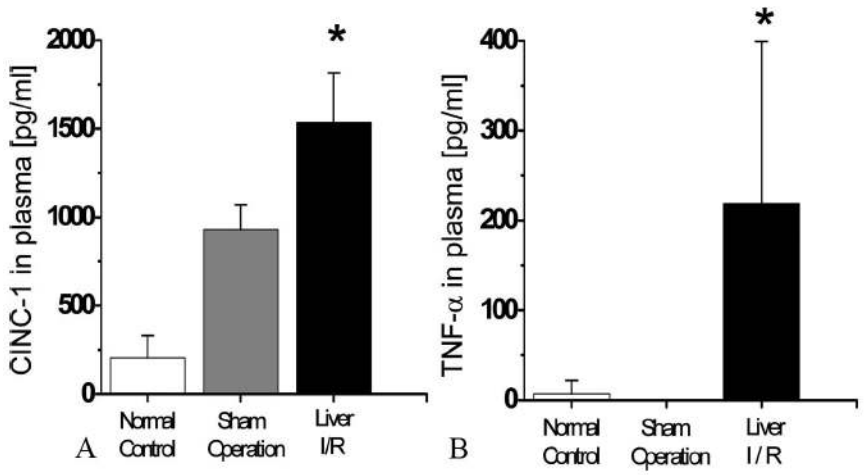

Figure 1.

A, tumor necrosis factor (*TNF*)-α concentration in the plasma. The TNF-α concentration in plasma of ischemia-reperfusion (*I/R*)-treated rats was significantly elevated at 219 ± 180 pg/mL (n = 6), whereas the cytokine had an average content of 7 ± 15 pg/mL in the plasma of normal controls (n = 5). TNF-α was not detected in plasma samples from sham operated rats. *B*, cytokine-induced neutrophil chemotactic factor (*CINC*)-1 concentration in the plasma. CINC-1 was significantly elevated in I/R-treated animals (1537 ± 279 pg/mL; n = 6) compared with both normal controls (204 ± 128 pg/mL; $p < .0001$) and sham-operated rats (930 ± 140 pg/mL; $p < .001$).

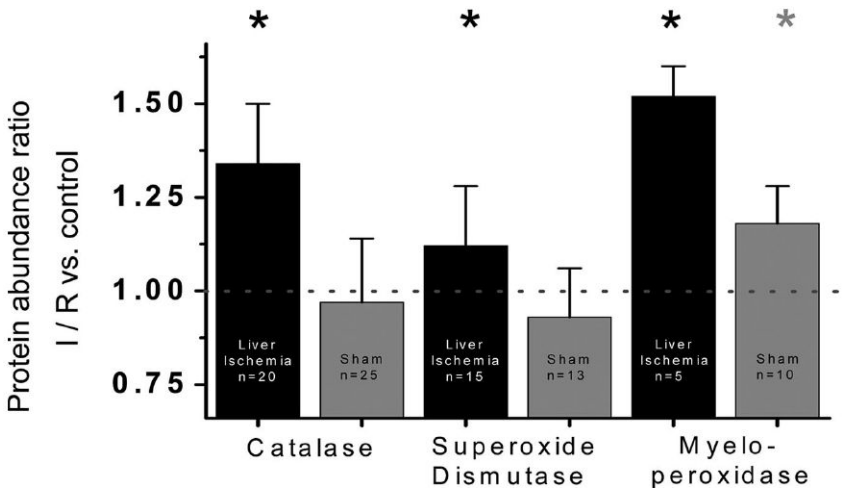

Figure 2.

Proteins of the oxidant system. The proteins catalase, myeloperoxidase, and superoxide dismutase were significantly elevated in alveolar epithelial type II cells from rats after liver ischemia-reperfusion (*I/R*) but not after sham operation. The bars represent the average iTRAQ protein abundance ratio; the error bars represent the overall mean and SD based on the individual peptide ratios.

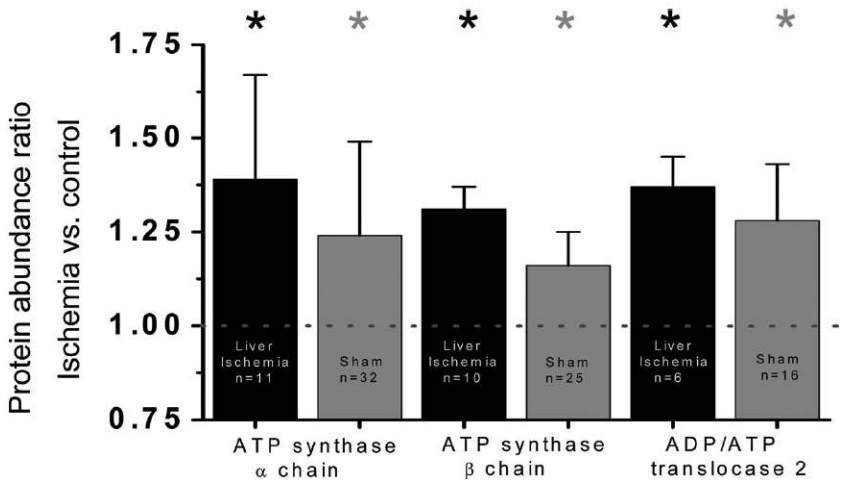

Figure 3.

Enzymes of the adenosine phosphate system. The three enzymes proteins adenosine triphosphate (*ATP*) synthase α chain, ATP synthase β chain, and adenosine diphosphate (*ADP*)/ATP translocase 2 were significantly elevated in rats after both liver ischemia-reperfusion and sham operation. The bars represent the average iTRAQ protein abundance ratio; the error bars represent the overall mean and SD based on the individual peptide ratios.

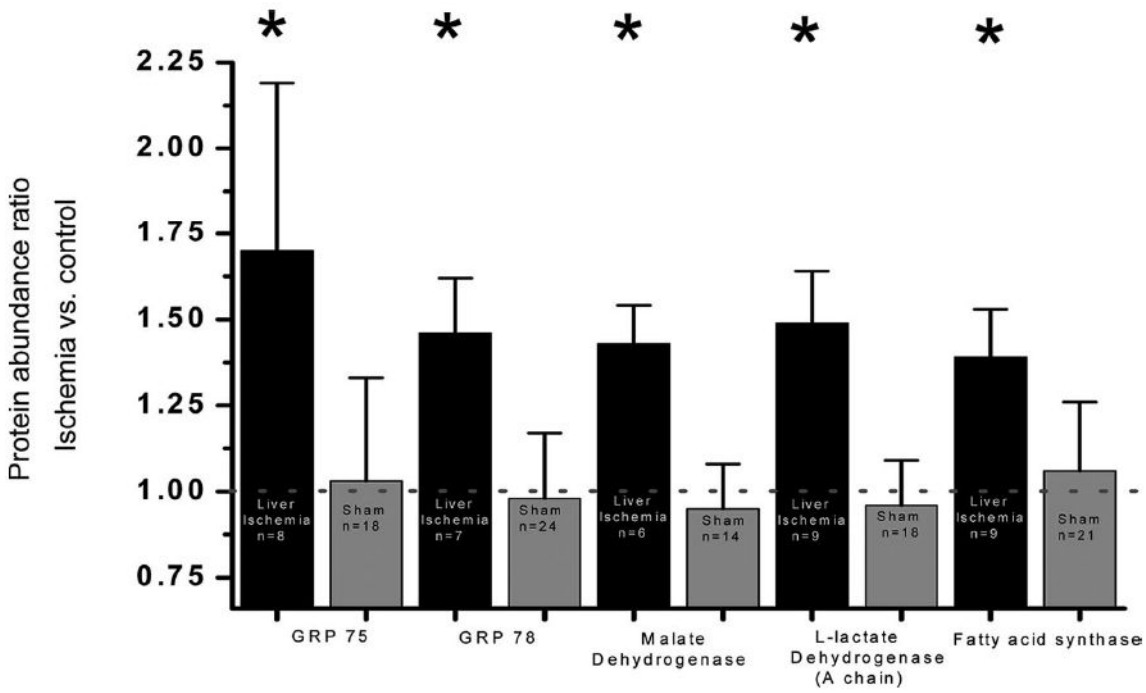

Figure 4.

Intracellular metabolic enzymes. There were significantly increased cellular contents in glucose regulating proteins (*GRP*) 75 and 78, malate dehydrogenase, L-lactate dehydrogenase (A-chain), and fatty acid synthase in rats after liver ischemia-reperfusion. In rats that had undergone sham operation, the content in these enzymes was essentially unchanged. The bars represent the average iTRAQ protein abundance ratio; the error bars represent the overall mean and SD based on the individual peptide ratios.

Table 1

Cells in bronchoalveolar lavage fluid

	Bronchoalveolar Lavage Cell Counts			Protein Concentration	
	Cells per ml BAL [$^{*}10^{5}$]	**Macrophages [%]**	**Neutrophils [%]**	**Others [%]**	**Per Sample [µg/ml]**
Normal controls	0.72 ± 0.04	96 ± 0.4	2 ± 0.4	2 ± 0.8	554.4 ± 353.2
Sham operation	1.34 ± 0.31*	90 ± 4.7	5 ± 3.0	3 ± 2.2	543.3 ± 328.2
Ischemia-reperfusion	2.12 ± 1.2*	49 ± 26*	48 ± 26*	4 ± 1.3	675.4 ± 62.1

* $p < .05$ vs. normal controls.

Table 2

Proteins of the oxidant system

Swiss Prot #	Protein Name	N Ischemia-reperfusion	Mean Ischemia-reperfusion/NC	STD Ischemia-reperfusion/ NC	P Ischemia-reperfusion/ NC	N Sham	Mean Sham/ NC	STD Sham/ NC	P Sham/ NC	P Ischemia-reperfusion/ Sham
P04762	Catalase (EC 1.11.1.6)	20	1.34	0.16	0.0001	25	0.97	0.17	0.4662	0.0001
P11247	Myeloperoxidase precursor (EC 1.11.1.7) (MPO)	5	1.52	0.08		10	1.18	0.10	0.0003	
P08228	Superoxide dismutase	15	1.12	0.16	0.0137	13	0.93	0.13	0.0580	0.0003

Table 3

Enzymes of the adenosine phosphate system

Swiss Prot #	Protein Name	N Ischemia-reperfusion	Mean Ischemia-reperfusion/NC	STD Ischemia-reperfusion/ NC	P Ischemia-reperfusion/ NC	N Sham	Mean Sham/ NC	STD Sham/ NC	P Sham/ NC	P Ischemia-reperfusion/ Sham
Q09073	ADP/ATP translocase 2 (Adenine nucleotide translocator 2)	6	1.37	0.08	0.0001	16	1.28	0.15	0.0000	0.3545
P15999	ATP synthase alpha chain, mitochondrial precursor (EC 3.6.3.14)	11	1.39	0.28	0.0010	32	1.24	0.25	0.0000	0.0830
P10719	ATP synthase beta chain, mitochondrial precursor (EC 3.6.3.14)	10	1.31	0.06	0.0001	25	1.16	0.09	0.0000	0.0006

Table 4

Cellular enzymes

Swiss Prot #	Protein Name	N Ischemia- reperfusion	Mean Ischemia-reperfusion/NC	STD Ischemia-reperfusion/NC	P Ischemia-reperfusion/ NC	N Sham	Mean Sham/ NC	STD Sham/ NC	P Sham/ NC	P Ischemia-reperfusion/ Sham
P06761	78 kDa glucose- regulated protein precursor (GRP 78)	7	1.46	0.16	0.0003	24	0.98	0.19	0.6582	0.0001
P11884	Aldehyde dehydrogenase, mitochondrial precursor (EC 1.2.1.3) (ALDH class 2) (ALDH1) (ALDH-E2)	11	1.43	0.17	0.0000	20	1.11	0.17	0.0091	0.0000
P00507	Aspartate aminotransferase, mitochondrial (EC 2.6.1.1)	10	1.42	0.09	0.0001	18	0.91	0.15	0.0192	0.0001
P16303	Carboxylesterase 3 precursor (EC 3.1.1.1)	5	1.42	0.18	0.0001	17	1.09	0.31	0.2393	
P12785	Fatty acid synthase (EC 2.3.1.85)	9	1.39	0.14	0.0001	21	1.06	0.20	0.2110	0.0001
P04642	L-lactate dehydrogenase A chain (EC 1.1.1.27)	9	1.49	0.15	0.0001	18	0.96	0.13	0.2348	0.0001
P11598	Protein disulfide-isomerase A3 precursor (EC 5.3.4.1)	5	1.64	0.19	0.0001	14	1.06	0.20	0.3006	0.0001
Q63081	Protein disulfide-isomerase A6 precursor (EC 5.3.4.1)	9	1.43	0.22	0.0004	16	0.98	0.17	0.6810	0.0001
P04785	Protein disulfide-isomerase precursor (EC 5.3.4.1)	10	1.32	0.12	0.0001	21	1.00	0.13	0.8892	0.0001

Table 5

Regulatory proteins

Swiss Prot #	Protein Name	N Ischemia-reperfusion	Mean Ischemia-reperfusion/NC	STD Ischemia-reperfusion/NC	P Ischemia-reperfusion/NC	N Sham	Mean Sham/NC	STD Sham/NC	P Sham/NC	P Ischemia-reperfusion/Sham
P26772	10 kDa heat shock protein, mitochondrial (Hsp10))	6	1.55	0.25	0.0028	11	0.92	0.19	0.1767	0.0001
P63101	Protein kinase C inhibitor protein 1	5	1.50	0.13	0.001	6	1.12	0.13	0.0713	0.0001
P63039	60 kDa heat shock protein, mitochondrial (Hsp60)	6	1.54	0.21	0.0014	15	0.96	0.15	0.3318	0.0001
P06761	78 kDa glucose- regulated protein precursor (GRP 78)	7	1.46	0.16	0.0003	24	0.98	0.19	0.6582	0.0001
P07150	Annexin A1 (Lipocortin I)	9	1.46	0.15	0.0001	21	1.23	0.26	0.0007	0.0118
Q07936	Annexin A2 (Lipocortin II)	5	1.33	0.20	0.02	20	1.31	0.36	0.0011	
O70371	Annexin A5 (Lipocortin V)	12	1.36	0.24	0.0003	13	1.10	0.16	0.0334	0.0036
P48037	Annexin A6 (Lipocortin VI)	11	1.45	0.15	0.0001	22	1.16	0.15	0.0000	0.0000
P05964	Calcyclin	7	1.26	0.16	0.0052	7	1.00	0.06	0.9799	0.0003
P18418	Calreticulin (CRP55) (Calregulin)	7	1.35	0.10	0.0001	16	0.98	0.12	0.5639	0.0001
P62630	Elongation factor 1-alpha 1	5	1.25	0.06	0.001	7	1.14	0.39	0.3956	0.51
P63017	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	11	1.07	0.09	0.0348	15	0.96	0.17	0.3607	0.0336
P34058	Heat shock protein HSP 90-beta (HSP 84)	10	1.09	0.20	0.1765	10	1.10	0.14	0.0417	0.8805
P67779	Prohibitin	7	1.15	0.10	0.0088	5	1.44	0.39		
P38647	Stress-70 protein, mitochondrial	8	1.70	0.49	0.0052	18	1.03	0.30	0.6810	0.0001
Q66HD0	Tumor rejection antigen gp96	15	1.28	0.18	0.0001	26	0.99	0.15	0.7216	0.0001
P62991	Ubiquitin	7	1.66	0.74	0.0573	9	1.00	0.27	0.9812	0.0113

Table 6

Structural proteins

Swiss Prot #	Protein Name	N Ischemia-reperfusion	Mean Ischemia-reperfusion/NC	STD Ischemia-reperfusion/ NC	P Ischemia-reperfusion/ NC	N Sham	Mean Sham/ NC	STD Sham/ NC	P Sham/ NC	P Ischemia-reperfusion/ Sham
P60710	Actin, cytoplasmic 1 (Beta-actin)	34	1.26	0.37	0.0002	34	1.19	0.31	0.0012	0.3330
Q6LED0	H3 histone	15	1.12	0.17	0.0172	20	0.93	0.20	0.1180	0.0022
P15865	Histone H1.2 (H1d)	21	1.02	0.12	0.3635	23	0.83	0.11	0.0000	0.0000
P02262	Histone H2A.1	7	1.47	0.20	0.0007	12	0.97	0.16	0.4916	0.0001
Q00715	Histone H2B	17	1.17	0.36	0.0660	26	1.04	0.29	0.4630	0.1770
P62804	Histone H4	15	1.07	0.26	0.2915	20	1.38	0.69	0.0258	0.0591
Q5BJY9	Keratin, type I cytoskeletal 18	7	1.44	0.20	0.0011	13	1.01	0.18	0.8511	0.0001
Q10758	Keratin, type II cytoskeletal 8	17	1.52	0.16	0.0001	36	0.95	0.18	0.0727	0.0001
P48679	Lamin A	28	1.22	0.11	0.0001	32	0.93	0.14	0.0078	0.0001
P70615	Lamin B1	9	1.50	0.19	0.0001	11	1.06	0.21	0.3848	0.0001
O35763	Moesin	14	1.20	0.18	0.0010	15	0.96	0.13	0.2559	0.0000
Q6URW6	Myosin-14	12	1.47	0.11	0.0001	21	1.22	0.21	0.0001	0.0074
Q62812	Myosin-9	27	1.47	0.22	0.0001	62	1.20	0.23	0.0000	0.0001
Q6XD99	Non-erythroid pectrin beta	10	1.45	0.12	0.0001	12	1.09	0.12	0.0280	0.0006
P30427	Plectin 1 (PLTN) PCN)	27	1.43	0.26	0.0001	28	1.30	0.26	0.0000	0.0322
P16086	Spectrin alpha chain	13	1.44	0.29	0.0002	22	1.02	0.29	0.7586	0.0001
Q63601	Tropomyosin	15	1.22	0.23	0.0021	20	0.86	0.20	0.0043	0.0001
Q5FWJ3	Vimentin	14	1.44	0.26	0.0001	23	1.00	0.37	0.9821	0.0001