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Identification and preliminary validation of novel biomarkers of acute hepatic ischaemia/reperfusion injury using dual-platform proteomic/degradomic approaches

S. I. SVETLOV[1,3], Y. XIANG[1,3], M. W. OLI[6], D. P. FOLEY[5], G. HUANG[1,3], R. L. HAYES[3,6], A. K. OTTENS[2,3], & K. K. W. WANG[2−4,6]

[1]*Laboratory of Molecular & Cellular Mechanisms of Injury,* [2]*Center for Proteomics and Biomarkers Research,* [3]*Departments of Neuroscience,* [4]*Psychiatry,* [5]*Surgery, University of Florida College of Medicine, Gainesville, FL 32610, USA and* [6]*Banyan Biomarkers, Inc., 12085 Research Drive, Suite 180, Alachua, FL 32615, USA*

Abstract
Hepatic ischaemia/reperfusion (I/R), a major cause of liver damage associated with multiple trauma, haemorrhagic and septic shock, and liver transplantation, contributes significantly to multiple organ failure. Development of novel sensitive biomarkers that detect early stages of liver damage is vital for effective management and treatment of ischaemic liver injury. By using high-throughput immunoblotting and cation–anion exchange chromatography/reversed-phase liquid chromatography-tandem mass-spectrometry, we identified several hepatic proteins, including argininosuccinate synthase (ASS) and estrogen sulfotransferase (EST-1), which were degraded in the liver and rapidly released into circulation during I/R injury. ASS accumulated in serum within 10 min, reached a steady state at 30 min, and persisted up until 3 h after reperfusion following 30 min of total hepatic ischaemia. EST-1 appeared rapidly in blood and attained maximum within 1 hour followed by a decline at 3 h of reperfusion. No ASS or EST-1 protein was detected in serum of control or sham operated rats. ASS and EST-1 exhibited greater sensitivity and specificity toward I/R liver injury as compared with alanine aminotransferase (ALT), an established marker of hepatocellular necrosis. In contrast, serum ASS and EST-1 were undetectable in rats with chronic alcoholic liver disease, while the levels of ALT protein were significantly increased. In addition, ASS, but not EST-1 or ALT accumulated in blood only 6 h after treatment with hepatotoxic combination of lipopolysaccharide and D-galactosamine. These data demonstrate the utility of ASS and EST-1 as novel sensitive and specific biomarkers of acute liver ischaemic injury for prospective clinical studies.

Keywords: *Biomarkers, rat, liver, ischaemia/reperfusion, injury, argininosuccinate synthase, estrogen sulfotransferase*

Correspondence: S. Svetlov, Laboratory of Molecular and Cellular Mechanisms of Injury, Center for Proteomics and Biomarkers Research, Department of Neuroscience, University of Florida College of Medicine, Gainesville, FL 32610, USA. Tel: 1-352-392-3204. E-mail: svetlov@mbi.ufl.edu

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Introduction

Liver failure due to various forms of hepatic injury is a significant source of overall mortality. There is a growing number of clinical conditions in the modern world where liver ischaemia followed by reperfusion (I/R) is a leading cause of hepatic injury: multiple and abdominal trauma including gunshot and explosion wounds, drug toxicity, haemorrhagic and septic shock, and graft failure after liver transplantation (Lemasters et al. 1997, Lemasters & Thurman 1997). Ischaemic liver injury is triggered by the release of reactive oxygen species (ROS), including peroxynitrite ($ONOO^-$) radicals by sinusoidal Kupffer cells and endothelial cells as well as adenosine triphosphate (ATP) depletion and acidification in hepatocytes. After reperfusion, the oxygen supply resumes, but liver injury increases severely due to microvascular dysfunction, profound inflammatory infiltration and cytokine/chemokine production (Jaeschke 1996, Jaeschke et al. 1996a,b). I/R results in hepatic cell death via both apoptosis and necrosis (Jaeschke 2003, Jaeschke & Lemasters 2003, Kim et al. 2003) accompanied by the release of cytosolic enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) into the extracellular milieu. Plasma ALT/AST have been commonly used as markers of liver injury including oxidative stress-induced hepatocellular damage. Unfortunately, the increase of these enzymes in the blood reflects mostly terminal stages of hepatocellular death, and therefore does not detect the early phases of hepatic injury for evaluation of magnitude of hepatocellular damage or assessing hepatic recovery. Thus, there is an explicit clinical need for better non-invasive biomarkers of ischaemia/reperfusion-induced liver injury that can result in early diagnostics and treatment.

A hallmark feature of apoptosis and necrosis is an early cleavage of several cellular proteins by activated caspases and calpains. As shown previously by a number of investigators including the present authors' group, caspase-3 and calpain-2 are major executioners of apoptotic and necrotic cell death, respectively, during ischaemic or traumatic injury. A signature of caspase-3 and calpain-2 activation in many tissues is a cleavage of several common proteins such as major cytoskeletal αII-spectrin (Sakon et al. 2002, Pike et al. 2004, Pineda et al. 2004, Ringger et al. 2004). We proposed that caspase-3 and, possibly calpain-2 activation results in the degradation of several liver-specific proteins that, when released, accumulate in the blood at early phases of hepatic damage due to impaired cell permeability.

To identify proteins differentially displayed in hepatic I/R samples versus control, we examined liver samples using two complementary proteomic techniques: (1) high-throughput immunoblotting (HTPI), and (2) combined cation–anion exchange chromatography-sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)/reversed-phase liquid chromatography tandem mass-spectrometry (CAX-PAGE/RPLC-MS-MS). Based on HTPI, we identified several hepatic proteins that are altered in liver tissue subjected to ischaemia/reperfusion injury such as argininosuccinate synthase (ASS), arginase-I (Arg-I), and squalene-synthase (SQS). The most relevant liver-specific proteins identified to date using CAX-PAGE/RPLC-MS-MS are estrogen sulfotransferase (EST), the liver isoform of glycogen phosphorylase (GP), hepatic enolase-1, carbamoyl-phosphate synthetase 1 (CPS-1), and glucose-regulated protein (GRP-58). Preliminary validation of plasma ASS and EST-1 assays demonstrated a greater sensitivity and specificity of these markers for ischaemia-reperfusion-induced liver injury as compared with ALT.

Materials and methods

Rat model of ischaemia/reperfusion injury

All procedures involving animals were performed according to guidelines from the National Institutes of Health and were approved by the IACUC of the University of Florida. Adult male Sprague–Dawley rats (220–250 g) were anaesthetized with 4% isoflurane for 4 min in a chamber until a surgical level of anaesthesia was achieved. Animals were placed on the heating pad and delivery of anaesthetic gas continued via a nose cone throughout the surgery. A midline approximately 3-cm-long laparotomy was made, and the liver was exposed. The hepatoduodenal ligament was dissected and occluded for 30 min using an atraumatic vascular clamp. After 30 min of normothermic ischaemia, recirculation of the blood through the ischaemic liver was achieved by removing the clamp for additional 10, 30 min, 1 and 3 h. At the end of reperfusion, blood was collected from heart; the liver was briefly perfused with cold phosphate-buffered saline (PBS) to remove residual blood and taken for analysis.

Chronic alcoholic liver model

Adult male Sprague–Dawley rats (180–200 g) were kept on nutritionally complete diet containing 36% ethyl alcohol for 14 weeks. An average blood alcohol level (BAL) of 150–175 mg dl^{-1} was achieved during the experimental period. Blood was collected from the heart; the liver was briefly perfused with cold PBS and taken for analysis.

LPS/D-galactosamine acute liver injury

Lipopolysaccharide (LPS, 011:B5, 50 µg kg^{-1}) plus D-galactosamine (D-Gal, 500 mg kg^{-1}) or saline were injected intraperitoneally (i.p.) in Sprague–Dawley rats as described previously with modifications (Jones et al. 1999, Dokladny et al. 2001). Blood was collected 15 min, 45 min, 1 h and 6 h after the treatment.

Liver tissue processing and sample preparation

Liver specimens were snap-frozen in liquid nitrogen after removal. Liver samples from I/R, naïve and sham-operated rats were homogenized on ice using a Polytron in radioimmuno-precipitation assay (RIPA) buffer consisting of PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, containing 0.1 mg ml^{-1} PMSF, 1 mM sodium orthovanadate, 5 mM ethylenediamine tetra-acetic acid (EDTA), 5 mM EGTA, and protease inhibitor cocktail (Roche Diagnostics Inc., Indianapolis, IN). For r-caspase-3 and r-calpain-2 treatment *in vitro*, livers obtained from intact (naïve) rats were homogenized in RIPA buffer consisting of PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 5 mM EDTA, 5 mM EGTA without protease inhibitors and centrifuged for 15 min at 10 000 rpm at 4°C. Supernatants were removed and the protein was measured using bicinechoninic acid (Pierce). Intact liver samples were treated *in vitro* with caspase-3 (Chemicon, Temecula, CA, specific activity 1 µg µl^{-1}) or calpain-2 (Calbiochem, San Diego, CA, 0.25 µg µl^{-1}) as described previously in detail (Haskins et al. 2004, Wang et al. 2004).

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High-throughput screen immunoblotting (HTPI)

HTPI is performed as previously described (Yoo et al. 2002, Malakhov et al. 2003). Briefly, hepatic protein (200 µg) was loaded in one big well across the entire width of the 13×10 cm, 4–15% gradient SDS-polyacrylamide gel (Bio-Rad Criterion IPG, Hercules, CA). This translates into approximately 8 µg per lane on a standard ten-well mini-gel. After separation, proteins were transferred to Immobilon-P membrane (Millipore, Billerica, MA). The membrane was blocked and clamped with a western blotting manifold that isolates 40 channels across the membrane. In each channel, a complex antibody cocktail was added and hybridized for 1 h at 37°C. Proteins were visualized with secondary goat anti-mouse antibody conjugated to Alexa680 fluorescent dye (Molecular Probes, Carlsbad, CA) and scanned at 700 nm using the Odyssey Infrared Imaging System. Each sample was run twice in two independent experiments. Protein bands from scanned images were measured by densitometry and expressed in arbitrary units. The values of protein bands increased in the I/R samples (up-regulation) were divided by the corresponding numbers in control (sham-operated rats) and fold-change is presented as mean$\pm$SEM preceded by the '+' sign (Table I). When the values in the I/R samples decreased compared with control group (down-regulation), then control values were divided by I/R numbers and fold-change is presented as mean$\pm$SEM preceded by the '−' sign (Table I). The values >100 indicates that the protein band was present only in the I/R samples (+), or detectable only in control samples (−). The similar calculations were done for caspase-3- and calpain-2-treated liver tissue versus intact samples (Table II, A and B).

Cation−anion exchange chromatography: SDS-PAGE/reversed-phase liquid chromatography tandem mass-spectrometry (CAX-PAGE/RPLC-MS-MS)

The entire experimental procedure is described by Haskins et al. (2004, 2005), Wang et al. (2004, 2005), and Ottens et al. (2005) in detail. Briefly, the LC system is set up to run two columns in line: S-Sepharose and Q-Sepharose. Samples were separated using gradient of mobile phase A (20 mM Tris-HCl) and B (20 mM Tris-HCl containing 1 M NaCl. Fractions were collected, concentrated and subjected to SDS-PAGE on BioRad Criterion Gels, 4–20% Tris-HCl 18 well gels. The samples were run in pairs, sham-operated (control) and I/R. Gels were stained with Coomassie-R250 and protein bands were selected for excision. Band excision, protein reduction, alkylation, digestion and extraction were performed as previously described by us in detail (Haskins et al. 2004, 2005, Wang et al. 2005, Ottens et al. 2005). The mass-spectrometry (MS-MS) was performed in a LCQ Deca XP, quadrapole ion trap mass spectrometer. The peptides were loaded on to a reverse phase column and eluted into the MS using an organic gradient and electrospray ionization. Resulting tandem mass spectra were correlated with tryptic peptide sequences extracted from a non-redundant mammalian protein database (NCBI) utilizing the Sequest algorithm (Haskins et al. 2004, 2005, Wang et al. 2004, 2005, Ottens et al. 2005). Peptide matches only of high spectral correlation were collected by use of DTASelect software data filtering, and IR versus sham liver proteomes were compared using Contrast software (Tabb et al. 2002). Specifically, peptide correlation values (X_{corr}) greater than 1.8, 2.5 and 3.5 for singly, doubly and triply charged peptides were selected, respectively. A minimum of two peptides was required for identification.

Table I. Quantitative analysis of proteins and protein breakdown products in liver tissue of rats subjected to hepatic ischaemia/reperfusion (I/R) compared with control rats. Liver tissues collected from control rats ($n = 4$) or I/R-treated rats ($n = 4$) were pooled. Proteins were separated by SDS-PAGE and analysed by HTPI using our custom 40 antibody mini-screen in duplicate (Runs 1 and 2) as described in the Materials and methods in detail. The HTPI images were captured (see Figure 2, A, B, as examples) and protein bands were quantified. The values of protein band increased in the I/R sample ($+$) were divided by the values in control samples. When the protein band in the I/R sample was decreased ($-$), the control sample value was divided by I/R. The results are presented as mean $\pm$ SEM of four independent measurements.

Lane	Protein ID	Research area	Predicted MW (intact protein)	Observed MW	Direction and fold change I/R versus control
27	ASS-24 kDa	mitochondria/urea cycle/nitric oxide	46	24	($+$) 14.9 $\pm$ 4.5
27	ASS-31 kDa	mitochondria/urea cycle/nitric oxide	46	31	($+$) >100
27	ASS-34 kDa	mitochondria/urea cycle/nitric oxide	46	34	($+$) >100
2	nNOS type I-67 kDa	nitric oxide synthase	155	67	($+$) 6.2 $\pm$ 2.3
8	Arginase-I	nitric oxide	35	37	($-$) 1.93 $\pm$ 0.2
9	Squalene synthase (SQS)	mitochondria/cholesterol synthesis	48	36	($+$) >100
16	MEK5	MAP kinase	50	50	($-$) 2.23 $\pm$ 0.09
16	MEK5	MAP kinase	50	21	($-$) >100
12	β-Catenin	tyrosine kinase	92	92	($+$) >100
31	Ninjurin	cell adhesion	22	21	($-$) 19.5 $\pm$ 3.9
13	α-Actinin	cytoskeleton/cell adhesion	104	113	($+$) >100

Results

Identification of hepatic proteins altered during I/R injury by high-throughput immunoblotting (HTPI)

Pooled liver samples from rats subjected to 30 min ischaemia followed by 30 min reperfusion and sham operated rats were examined using HTPI. Intact liver tissues were treated *in vitro* with recombinant caspase-3 or calpain-2 and analysed by HTPI using the same antibody mini-screen. Initially, we designed a custom mini-array of 40 antibodies from a list of over 1000 antibodies available at BD Pharmingen, (San Jose, CA). We selected proteins that are known to be expressed predominantly in the liver and play important roles in hepatic pathophysiology, or are important components of cell cytoskeleton integrity including hepatic cells. The results are presented as images of 40 antibody immunoblotting mini-screen of control (sham-operated) and I/R samples (Figure 1, A and B), *in vitro* caspase-3-treated intact samples and calpain-2 treated samples (Figure 1, C and D).

Protein bands of interests were quantified in the I/R samples versus sham operated samples, with the results presented in Table I. The same analysis has been performed for caspase-3- and calpain-2-treated samples versus intact liver tissue (Table II). I/R induced up-regulation of various proteins in liver tissue (e.g. ASS, SQS, β-catenin) including a concomitant accumulation of protein breakdown products (e.g. ASS, nNOS), while a modest down-regulation of intact arginase-I was observed without accumulation of detectable cleavage fragments on HTPI. As can be seen from Figure 1 and Tables I and II, hepatic ASS was up-regulated, predominantly due to a

Table II. Quantification of proteins and protein breakdown products in normal rat liver tissue treated *in vitro* with caspase-3 (A) and calpain-2 (B) related to untreated controls. Liver tissues from intact rats ($n = 4$) were digested *in vitro* with the recombinant caspase-3 or calpain-2 as described in the Materials and methods in detail. Proteins were analysed by HTPI using our custom 40 antibody mini-screen. The samples were run in two independent experiments in duplicate. The HTPI images were captured (Figure 2, C, D) and protein bands were quantified. The analysis and presentation of the most prominently altered protein bands in caspase-3 digested samples versus intact liver tissue was performed exactly as described in Table I.

A

Lane	Protein ID	Research area	Predicted MW	Observed MW	Direction and fold change caspase-3 versus control
27	ASS-21 kDa	mitochondria/urea cycle/nitric oxide	46	21	(+) >100
27	ASS-24 kDa	mitochondria/urea cycle/nitric oxide	46	24	(+) 2.57±0.18
27	ASS-31/34 kDa	mitochondria/urea cycle/nitric oxide	46	31/34	(+) >100
2	nNOS type I-67 kDa	nitric oxide synthase	155	67	(+) 7.73±2.9
16	MEK5	MAP kinase	50	50	(+) 2.57±0.15
16	MEK5	MAP kinase	50	21	(+) 8.64±2.66
12	β-Catenin	tyrosine kinase	92	92	(+) >100
31	Ninjurin	cell adhesion	22	21	(−) 3.22±0.43

B

Lane	Protein ID	Research area	Predicted MW	Observed MW	Direction and fold change calpain-2 versus control
2	nNOS type I-67 kDa	nitric oxide synthase	155	67	(+) 5.55±2.0
16	MEK5	MAP kinase	50	45	(+) >100
16	MEK5	MAP kinase	50	24	(+) >100
31	Ninjurin	cell adhesion	22	21	(−) >100

concomitant accumulation of caspase-3, but not calpain-2 mediated cleavage fragments.

In addition to the liver-specific proteins, there was substantial degradation of ninjurin, a non-specific cell adhesion protein, in I/R hepatic tissue compared with sham operated rats (Figure 1, A and B). Ninjurin was degraded mostly via calpain-2-dependent cleavage and, to a lesser extent, via caspase-3 activation (Figure 1, C and D). The p50 MEK5 component of the MAP kinase signalling cascade was significantly degraded, apparently through both caspase-3 and calpain-2 dependent cleavage with the appearance of p21 and p24 fragments, respectively (Figure 1 and Tables I and II). Interestingly, β-catenin and α-actinin, both cell adhesion related proteins, accumulated in the liver during I/R, and appeared to be associated with caspase-3 activity (Figure 1, A–C). Although Arg-I has been considered previously as a potential candidate biomarker of hepatocellular injury, comprehensive studies of its diagnostic value in liver ischaemia/reperfusion have not been performed (Ikemoto et al. 2001). In contrast, ASS and SQS are liver-specific proteins,
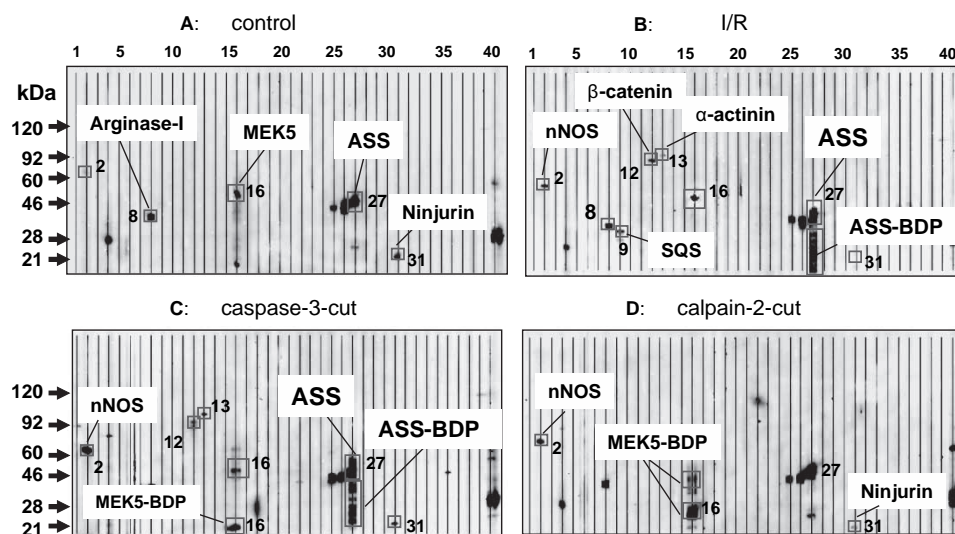
Figure 1. High-throughput immunoblotting (HTPI) of liver samples using a custom 40 antibody mini-screen array. Liver tissue obtained from four rats in each experimental group was pooled and processed as described in the Materials and methods in detail. A, Control rat livers; B, rat livers subjected to 30 min of normothermic ischaemia followed by 30 min of reperfusion (I/R, 30/30); and C, D, control rat livers treated with recombinant caspase-3 (C) or calpain-2 (D) *in vitro*. Representative blot from two runs of identical samples is shown. Squires depict proteins up or down regulated in I/R, caspase-3- and calpain-2-treated livers versus control. The numbers indicate the lane number on the screen. Lanes: 2, nitric oxide synthase (nNOS); 8, arginase-I; 9, squalene synthase (SQS); 12, β-catenin; 13, α-actinin; 16, MEK5; 27, ASS; 31, ninjurin. ASS-BDP, ASS breakdown products; MEK5-BDP, MEK5 breakdown products.

which have not been recognized previously as liver ischaemia/reperfusion injury biomarkers.

Analysis of hepatic proteins degraded in response to I/R injury using CAX-PAGE/RPLC-MS-MS

Proteins in control (sham-operated, C) and I/R samples (T) were separated using biphasic ion-exchange chromatography. Protein fractions were collected, paired-up and subjected to SDS-PAGE with Coomassie blue R-250 staining.

As can bee seen in the differential display (Figure 2), there are a number of proteins that are degraded in the liver in response to ischaemia/reperfusion. A set of down regulated proteins (greater than a twofold) were selected (squires) after quantification by Phoretix 1D software. Protein bands were excised from control (C) and treated (T) lanes, and digested with trypsin as described in the Materials and methods. Digests were analysed using reversed-phase liquid chromatography online with tandem mass spectrometry (RPLC-MS-MS). Resulting tandem mass spectra were correlated with tryptic peptide sequences extracted from a non-redundant mammalian protein database (NCBI) utilizing the Sequest algorithm. Peptide matches only of high spectral correlation were collected by use of DTASelect software data filtering, and IR versus sham liver proteomes were compared using Contrast software. Identification and analysis of the most relevant hepatic proteins performed so far are presented in Table III.
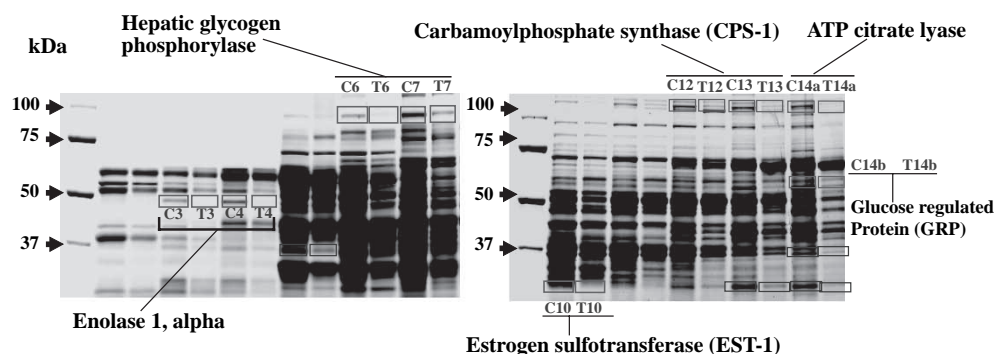
Figure 2. Differential SDS-PAGE display of protein fractions collected after combined cation −anion exchange (CAX) chromatography. The CAX fractions obtained from control (C) and I/R (T) samples were paired up and loaded side-by-side on SDS-PAGE. Proteins with differential expression were quantified using Phoretix 1D software. The numbers represent fraction number of control (C) or I/R sample, respectively. Portions of two gels containing fractions 1–15 are shown. Labeled boxes depict differentially displayed proteins already identified by RPLC-MS-MS. Proteins identity determined by RPLC-MS-MS is shown close to the red boxes. Unlabeled boxes indicate proteins to be identified. EST-1, oestrogene preferring sulfotransferase 1E; CSP-1, carbamoyl phosphate synthase 1; ACL, ATP citrate lyase; GRP, glucose-regulated protein p58.

All proteins identified as down regulated in the I/R samples versus controls, exhibited the predicted molecular weight, except carbamoyl-phosphate synthetase 1 (CPS-1), which was identified as 120 kDa protein (predicted 165 kDa) in control samples. Liver glycogen phosphorylase (GP), estrogen sulfotransferase (EST-1), and surprisingly glucose-regulated protein (GRP) demonstrated the highest abundance among degraded proteins. From this list of proteins, CPS-1 has been examined previously as potential biomarker of hepatocellular injury, although comprehensive studies of their diagnostic value in liver ischaemia/reperfusion have not been performed (Ozaki et al. 1994). The potential diagnostic value of other protein markers, particularly EST-1 and GRP, are until now unknown.

Selection of biomarker candidates and further analysis of their hepatic expression

Next, we selected proteins that were identified by using both HTPI and CAX- PAGE/ RPLC-MS-MS for further examination. Based on preliminary data, we selected ASS and EST-1 as lead biomarker candidates for a panel of liver I/R-induced injury. Also, we examined hepatic expression of cytoskeletal protein α-II spectrin and the accumulation of its degradation products (SBDPs) as a 'standard' to estimate contribution of caspase-3 or calpain-2 pathways following I/R. Appearance of SBDPs is an established characteristic of αII-spectrin cleavage by caspase-3 (SBDP150i and 120) and/or calpain-2 (SBDP145) in several tissues (Wang 2000), Indeed, after 30 min of hepatic ischaemia followed by 30 min of reperfusion, expression of intact α-II-spectrin (280 kDa) was decreased with concomitant accumulation of spectrin breakdown products SBDP 150i, SBDP145 and SBDP120 (Figure 3A).

I/R induced a significant increase of ASS breakdown products (approximately 24 and 31 kDa) within 10 min with a further increase at 30 min after initiation of reperfusion (Figure 3, B), consistent with the data obtained using HTPI (Figure 1, B). Hepatic expression of intact arginase-I was not significantly altered 30 min after

Table III. Results of CAX-PAGE/RPLC-MS-MS differential analysis of I/R samples versus sham-operated controls. Select protein bands indicating down regulation (greater than a twofold change) after I/R were identified by RPLC-MS-MS. Presented in the table are the protein name, gi reference number, predicted and observed protein mass, the number of peptides identified (C_{pep}, T_{pep}) and the percent sequence coverage ($C\%$, $T\%$) for control and I/R samples. Peptide number is used to validate decreased regulation.

Band	Accession number	Protein	Predicted MW	Observed MW	C_{pep}	$C\%$	T_{pep}	$T\%$
4	gi:6978809	enolase 1, alpha	47.5	47	4	11		
6	gi:11560087	liver glycogen phosphorylase	97.9	99	2	2.1		
7	gi:11560087	liver glycogen phosphorylase	97.9	99	8	17.5	2	4.8
10	gi:6981594	estrogen sulfotransferase	35.4	35	2	7.1		
12	gi:8393186	carbamoyl-phosphate synthetase 1	164.6	120	2	1.5		
13	gi:8393186	carbamoyl-phosphate synthetase 1	164.6	120	3	2.3		
	gi:8392839	ATP citrate lyase	121.5	120	2	2.8		
14A	gi:8393186	carbamoyl-phosphate synthetase 1	164.6	120	5	4.1		
14B	gi:8393322	glucose regulated protein, 58 kDa	57	56	6	13.1		

reperfusion; however, accumulation of protein breakdown fragments (approximately 15–18 kDa) was increased at both 10 and 30 min of reperfusion compared with sham-operated rats (Figure 3, C). These products were not displayed on HTPI images, probably due to low molecular weight limitations (Figure 1). Surprisingly, there was no significant decrease in intact EST-1 found in the I/R samples within 30 min of reperfusion (Figure 3, D). However, significant amounts of EST-1 breakdown products did accumulate within 30 min after reperfusion (Figure 3, D).

Validation of diagnostic utility of ASS and EST-1

Preliminary validation of diagnostic values of novel hepatic biomarkers was performed by measuring ASS and EST-1 levels in blood after 30 min of reperfusion following
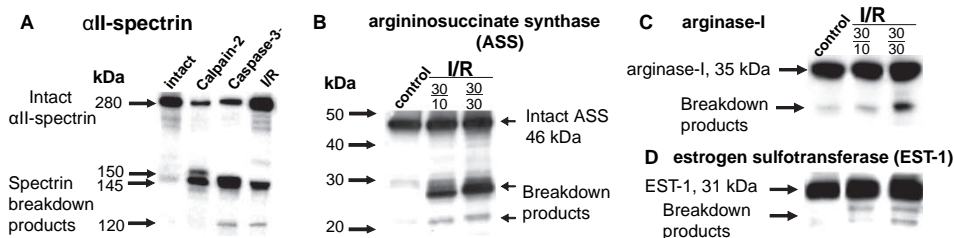

Figure 3. Hepatic expression of cytoskeletal αII-spectrin, liver-specific marker proteins, and their breakdown products following liver ischaemia/reperfusion injury. Liver samples were obtained from intact animals, control (sham operated) or rats subjected to 30 min hepatic ischaemia (I/R) followed by 10- and 30 min reperfusion. Intact liver tissue lysates were treated *in vitro* with recombinant caspase-3 or calpain-2 (A) as described in the Materials and methods in detail. Hepatic proteins (25 μg) were analysed by SDS-PAGE/ western blotting with antibodies to non-erythroid αII-spectrin (A), argininosuccinate synthase (B), arginase-I (C) and estrogen sulfotransferase (D), and visualized using enhanced chemiluminescence (ECL). A, Accumulation of αII-spectrin breakdown products in I/R livers similar to caspase-3 (120 kDa) and calpain-2 (145 kDa)-dependent cleavage fragments; B, appearance of caspase-3-dependent ASS cleavage fragments within 10 min after reperfusion; and C, D, hepatic levels of arginase-I and EST-1 after 10 and 30 min of reperfusion. Representative western blot images from three different caspase-3 and calpain-2 treatments of pooled intact liver tissues (A) and from four experimental rats in each group of I/R injury are shown.

30 min of warm hepatic ischaemia in rats. No plasma ASS or EST-1 was detected in intact (N1, N2), sham-operated (S1, S2) or chronic alcohol-treated rats (A1, A2 A3) (Figure 4, A). Intact ASS (46 kDa) and EST-1 (31 kDa) proteins accumulated in blood of rats subjected to 30/30 min of warm ischaemia/reperfusion (Figure 4, A). Plasma levels of ALT protein (57 kDa predicted MW) were not changed significantly in I/R and sham-operated rats; in contrast, plasma ALT protein was increased substantially in chronic alcohol rats (Figure 4, A).

Blood levels of ASS, EST-1 and ALT proteins were assessed in rats treated with LPS/D-galactosamine, another model of acute hepatocellular injury. ASS, but not EST-1 accumulated in blood only at 6 hour after i.p. injection of compounds (Figure 4, B). The levels of ALT protein did not change significantly within 6 h after treatment (Figure 4, B).

Finally, we examined ASS and EST-1 during time-course of reperfusion after 30 min of total ischaemia. Blood levels of intact ASS (46 kDa) rapidly attained a steady-state within 30 min, and persisted up until 180 min after initiation of reperfusion (Figure 5, A). In contrast, accumulation of ASS breakdown products in circulation (60–180 min) has been delayed compared with liver tissue (Figure 3, B). Blood levels of EST-1 rose quickly and attained maximum values within 30–60 min
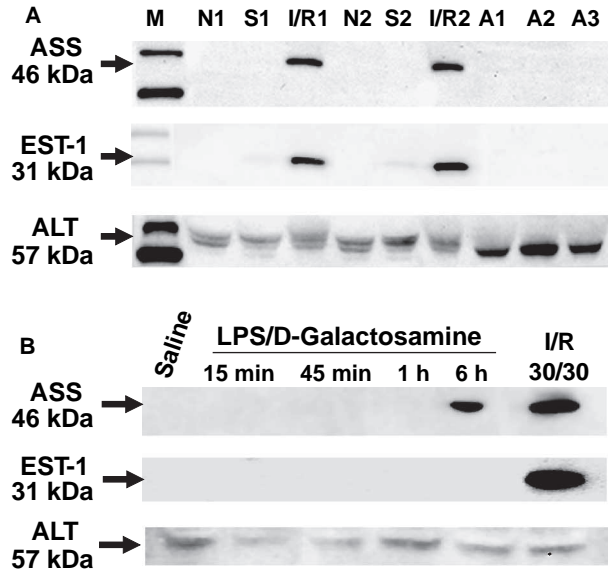

Figure 4. Accumulation of biomarkers of liver injury in blood after hepatic ischaemia/reperfusion, chronic alcoholic disease and acute endotoxic liver injury. A, Blood was withdrawn from rat heart after 30 min of ischaemia followed by 30 min of reperfusion and from chronic alcoholic rats as described in the Materials and methods. B, Rats were treated with LPS/D-galactosamine or saline as described in the Materials and methods. Serum or plasma was collected and equal volumes (10 μl) were processed as described in the Materials and methods in detail. Proteins were separated by SDS-PAGE and immunoblotted with antibody against ASS, EST-1 or alanine aminotransferase ALT. Membranes were developed by ECL, and images were scanned. Representative blots out from four or five performed using at least three different experiments are shown (A). N, intact, naïve rats (N1, N2, $n = 2$); S, sham operated rats (S1, S2, $n = 2$); I/R, 30-min ischaemia followed by 30-min reperfusion rats (I/R1, I/R2, $n = 2$); and A1, A2, A3, chronic alcoholic rats ($n = 3$). Representative blot out from three performed is shown for LPS/D-Gal treatment using three rats for each time point; the I/R sample (30/30) was included for comparison (B).

followed by a significant decline at 3 h (Figure 5, B). Plasma and serum patterns of ASS exhibited essentially the similar profiles, while serum EST-1 appeared to increase faster than plasma levels suggesting possible contribution of platelet and/or leukocyte EST-1 released into circulation (Figure 5, C, D).

Discussion

For the discovery of novel biomarkers of liver ischaemia/reperfusion-induced injury, we developed and implemented a liver proteome degradomics approach. Generally, the degradomics methodology is based on the notion that many types of injury to various organs and tissues, including traumatic, ischaemic or toxic insult, are mediated via apoptotic and/or necrotic pathways, and as such are accompanied by cleavage (degradation) of several tissue-specific proteins as well as common proteins, such as cytoskeletal αII-spectrin. Thus, identification by differential display of proteins that are degraded (cleaved) in injured tissue versus control reveal molecules, which can be released outside the cells into circulation as the full size (intact) proteins and/or in the form of protein breakdown products (BDPs) and serve as potential biomarkers. Specifically, we proposed that proteins subjected to degradation or cleavage, preferentially by activated caspase-3 and/or calpain-2 upon I/R injury and/or protein BDPs, can be accumulated in circulation at early phases of liver damage due to impaired hepatic permeability. Previously, based on degradomics approaches, our
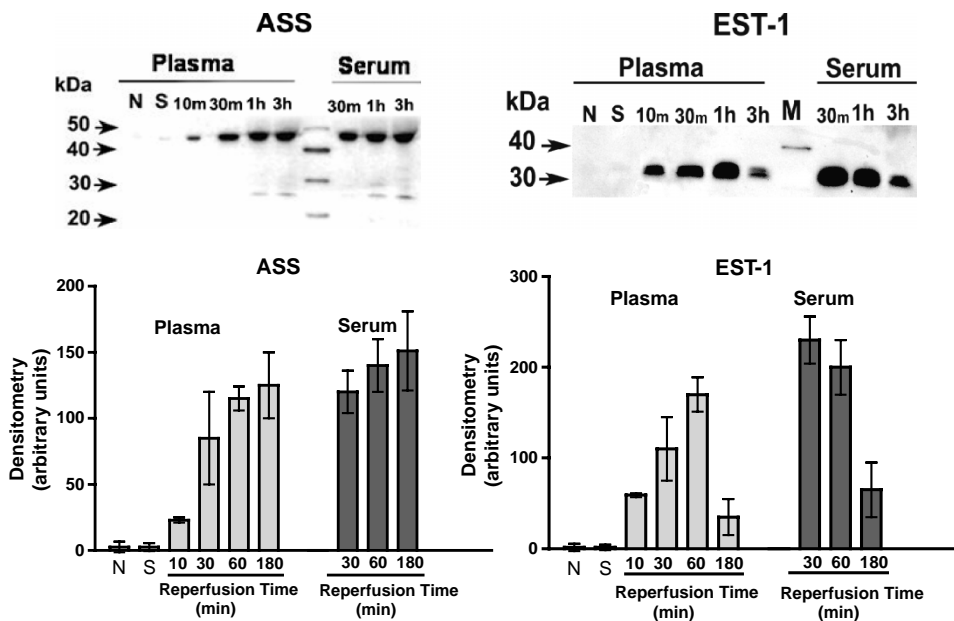

Figure 5. Time-dependent accumulation of the blood ASS and EST-1 after I/R in rats. Blood was withdrawn from rat heart following 30-min ischaemia followed by different times of reperfusion as described in the Materials and methods. Proteins were separated by SDS-PAGE/western blot with antibody against ASS (A) and EST-1 (B). Images were captured and protein bands were calculated using ImageJ software (C, D). Representative blots from five performed using at least four different experiments are shown. N, intact, naïve rats ($n = 5$); S, sham operated rats ($n = 4$); I/R, 30-min ischaemia followed by 10–180 min of reperfusion ($n = 4$).

group developed HTPI and CAX-PAGE/RPLC-MS-MS and used these techniques for the first time for discovery of biomarkers of traumatic brain injury (Wang et al. 2004, 2005).

The present studies clearly demonstrate the utility of degradomic approach for development of novel biomarkers of ischaemic liver injury. Based on HTPI, we identified several hepatic proteins, which were altered in liver tissue subjected to ischaemia/reperfusion injury such as mitochondrial enzymes argininosuccinate synthase (ASS), arginase-I (Arg-I), and squalene-synthase (SQS), Hepatic ASS was up-regulated with concomitant accumulation of caspase-3 mediated degradation fragments, while SQS and Arg-I were up- and down-regulated, respectively, without detectable appearance of breakdown products on mini-screen image (Figure 1 and Tables I and II).

CAX-PAGE/RPLC-MS-MS revealed a number of protein bands down regulated in I/R liver tissue compared with sham operated rats (Figure 2). Select down regulated protein bands exhibiting a twofold or greater decrease were identified (red boxes) (Figure 2 and Table III). Several other protein bands that were also altered significantly (indicated by unlabeled boxes). A complete analysis of differential proteins is under way and will be presented in a following manuscript.

While carbamoyl-phosphate synthase (CPS-1) has been reported to be a potentially useful marker of hepatitis (Ozaki et al. 1994), the data regarding enolase-1 or liver glycogen phosphorylase (GP) as specific biomarkers associated with hepatic injury are insufficient and controversial. In addition, glucose-regulated protein p58 has been shown to play an important role in toxic liver damage including alcoholic hepatitis, though its significance as potential biomarker has not been studied. In contrast, the roles for estrogen sulfotransferase (EST-1) in hepatic damage including oxidative stress-induced injury were not recognized previously.

Recently, the potential diagnostic value for Arg-I (Ikemoto et al. 2001, Aldemir et al. 2003) and CPS-1 (Lim et al. 1992) was reported in rat liver ischaemia/reperfusion. While Arg-I and CPS-1 appear to be promising candidates as biomarkers for liver I/R injury, the comprehensive studies of these enzymes have not been performed. In contrast, ASS and EST-1 are liver-specific proteins, which have not been recognized previously as liver ischaemia/reperfusion injury biomarkers. Our preliminary data (not shown) indicated that the sensitivity of Arg-1 and, especially CPS-1 is significantly lower than ASS. Thus, ASS and EST-1 were selected for further characterization and validation of novel biomarkers of I/R liver injury.

Caspase-3 and calpain-2 are major executioners of apoptotic and necrotic cell death, respectively, during ischaemia or traumatic injury (Wang 2000, Wang et al. 2004, 2005, Yang et al. 2004). A signature of caspase-3 and calpain-2 activation in many tissues is a cleavage of several common proteins such as major cyto-skeletal αII-spectrin (Pineda et al. 2004, Ringger et al. 2004). I/R stimulated a cleavage of αII-spectrin via both caspase-3 and calpain-2 dependent pathways as indicated by accumulation of αII-spectrin degradation products (SBDP) (Figure 3, A). These findings are in great accordance with our previous data on appearance of SBDP in cerebrospinal fluid (CSF) after traumatic brain injury (Ringger et al. 2004). Conventional Western blot analysis of hepatic ASS expression showed a cleavage pattern upon I/R injury (Figure 3, B) consistent with data obtained using HPTI mini-screen with a detectable accumulation of degradation products within 10 min after reperfusion. Similarly, a slight degradation of hepatic arginase-I

and EST-1 was found within the same time-frame following reperfusion that was not detected by HPTI due to a lower molecular weight of cleavage fragments (Figure 3, C and D).

In the course of research on biomarkers, our laboratories have developed several criteria for biomarker development. Useful biomarkers such as ASS and EST-1 should employ readily accessible biological material such as blood, urine, saliva or CSF, correlate with the magnitude of injury and resulting functional deficits, possess high sensitivity and specificity, have a rapid appearance in biological fluids and be released in a time-dependent sequence after injury. Ideally, biomarkers should employ biological substrates unique to the liver and, at the same time, provide information on injury mechanisms, a criterion that is often used to distinguish biochemical mechanistic markers from surrogate markers of injury since surrogate markers usually do not provide information on injury mechanisms. The use of ASS and EST-1 as biomarkers for liver I/R injury confers a number of important advantages over existing biomarkers. First, ASS and EST-1 proteins are expressed predominantly in the liver and, to a much lesser extent, kidney. Second, ASS and EST-1 are not found in erythrocytes. Thus, assessments of ASS and EST-1 in serum or plasma are not confounded by red blood cell haemolysis. Lastly, ASS is a limiting step in biosynthesis of both urea in the liver and nitric oxide, thereby providing a perfect 'pathogenesis-dependent' marker linking ischaemia and liver function, while EST-1 indicates the conjugative ability of hepatocytes per se.

No blood ASS and EST-1 were detected in intact, sham-operated or chronic alcohol-treated rats, while ASS and EST-1 rapidly accumulated in plasma and serum after 30 min of reperfusion (Figure 4, A). In contrast, plasma levels of ALT were unchanged during I/R and were significantly elevated in alcohol-treated rats (Figure 4, A). In the LPS/D-galactosamine-induced model of acute liver damage ASS appeared in blood only 6 h after injection (Figure 4, B), while after reperfusion following 30-min ischaemia, ASS accumulated in circulation within 10 min, rapidly attained a steady state within 30 min, and persisted up until 180 min after initiation of reperfusion (Figure 5). In addition, accumulation of ASS breakdown products in circulation (60–180 min) has been delayed compared with liver tissue. Blood EST-1 was not detectable during 6 h after LPS/D-galactosamine injection (Figure 4, B), whereas during I/R blood levels of EST-1 rose within minutes and attained maximum within 60 min followed by significant decline at 3 h (Figure 5). Plasma levels of ALT protein were not changed during 6 h after LPS/D-galactosamine treatment, in accordance with previous data showing that serum ALT activity begin to rise at 12 h after treatment peaking at 24 h (Namisaki et al. 2006). Thus, ASS was more sensitive than ALT in detecting of LPS/D-Gal acute liver injury. Hence, its release in blood within minutes (not hours) after I/R indicated higher sensitivity of this marker for detection of I/R-induced liver damage than during acute LPS/D-Gal hepatotoxicity. However, a potential significance of ASS as a marker of other types of acute liver injury requires further investigation.

In conclusion, based on two platforms of proteomic/degradomic technology, novel biomarkers of liver ischaemia/reperfusion induced injury have been discovered. Preliminary validation of the most promising candidates ASS and EST-1 has been performed by measuring blood levels of these proteins and has demonstrated a higher sensitivity and specificity of ASS and EST-1 over established marker of hepatocellular injury ALT. We are currently developing sandwich ELISA diagnostic

tests for measurement of ASS and EST-1 in biological fluids. Design of a comprehensive panel of novel and specific biomarkers of I/R injury for subsequent clinical trials will greatly improve diagnostics and management of clinical conditions accompanied by ischaemic hepatic damage. Moreover, further studies of these biomarkers may provide more information on biochemical and molecular mechanisms contributing to liver injury, recovery of function and/or potential targets for novel therapeutic strategies.

Acknowledgements

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