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Hypoxia and reoxygenation of primary human hepatocytes induce proteome changes of glucose metabolism, oxidative protection and peroxisomal function

CHRISTOPH W. STREY[1], JOHANNES GESTRICH[1], TOBIAS BECKHAUS[2], ROSA MARIA MARQUEZ-PINILLA[1], ELSIE OPPERMANN[1], CHRISTIAN MÖNCH[1], JOHN D. LAMBRIS[3], MICHAEL KARAS[2] and WOLF O. BECHSTEIN[1]

[1]Department of General and Vascular Surgery, Johann Wolfgang Goethe-University Frankfurt, Medical School, Frankfurt; [2]Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe University Frankfurt, Frankfurt, Germany; [3]Department of Pathology and Laboratory Medicine, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104, USA

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Abstract. Protective hepatocellular responses to a hypoxic challenge are crucial to preserve liver function. The knowledge of affected metabolic functions could help assess and enhance hepatic ischemic tolerance. Here we studied adaptive mechanisms in human hepatocytes after hypoxia and reoxygenation using a proteomic approach. Proteins from primary hepatocytes were extracted after 6 h of hypoxia and 24 h of reoxygenation. The proteome was analyzed by 2D-electrophoresis. Densitometry and mass spectrometry (MALDI-TOF-MS) were used for protein identification. Two hundred and sixty-two spots were differentially analyzed and 33 spots displayed significant differences between hypoxic and normoxic cells. Seventeen proteins were identified by mass spectrometry. After hypoxia and reoxygenation the UTP-glucose-1-phosphate uridyltransferase, phosphoglycerate kinase1, fructose-1,6-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, thiosulfat-sulfurtransferase, thioredoxin peroxidase, peroxiredoxin III, and annexin A2 proteins were down-regulated. An increased expression was found for carbamoyl phosphate synthetase I, heat shock 70 kDa protein5, phosphoenol-pyruvate carboxy-kinase, catalase isoform2, peroxiredoxin II, glutathione S-transferase, hydroxyacid oxidase1, and F1-ATP synthase, α subunit1. Hepatocellular adaptation to hypoxia and reoxygenation involve glucose metabolism, peroxisomal functions, and oxidative stress protection. The identified proteins can serve as possible diagnostic targets to monitor hepatic hypoxic tolerance e.g. in the context of liver surgery and transplantation.

Correspondence to: Dr Christoph W. Strey, Department of General and Vascular Surgery, Johann Wolfgang Goethe-University Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany
E-mail: strey@gmx.de

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Introduction

Hepatic ischemia/reperfusion-induced injury (IRI) is a concern in various clinical settings, including liver transplantation (LTX) and general liver surgery. Cold preservation of liver grafts is associated with prolonged ischemia and followed by reoxygenation during the reperfusion phase. The process of IRI initiates many pathophysiological mechanisms with damaging consequences for the liver graft. The length of cold ischemia time is a determinant for the degree of graft damage during the early and late phase of reoxygenation. Furthermore, other clinical factors such as donor age, lipid and energy status as well as fatty degeneration of the organ contribute to the magnitude of cold storage and reperfusion insult. All these factors may lead to early and late liver graft dysfunction. The adaptive mechanisms that are initiated due to IRI also at the cellular level of the hepatocytes, which enable the liver graft to regain function are still not well defined.

A proteomic study of the process of hypoxia and reoxygenation (HR) in primary hepatocyte cultures in comparison with hepatocytes under normoxia (N) has not been undertaken yet. The global approach to changes of the proteome could contribute to the understanding of the adaptive mechanisms initiated under HR on a cellular level. Proteomics is a powerful methodology that allows assessment of global alterations in biological systems at the protein level (1,2). Proteomic techniques have been employed to investigate the *in vivo* and *in vitro* proteome response of cells and animal models to drugs, diseases and trauma (3-6).

By using comparative proteome analysis via two-dimensional gel electrophoresis (2-DE) between hepatocytes cultured under normoxic conditions and hepatocytes undergoing a sequence of prolonged HR, differentially expressed proteins can be identified by matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-

MS) and database searching. This approach enabled us to show that a sequence of HR induced protein expression changes in three major functional protein groups in hepatocytes. Among these were proteins from the context of glucose metabolism, oxidative and cellular stress protection and peroxisomal function. The identified cellular functions represent relevant targets for the adaptation processes under the influence of HR.

Materials and methods

Isolation of primary hepatocytes and cell culture. Normal human liver tissue was obtained from liver resection. Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. Human hepatocytes were isolated by a two-step collagenase perfusion technique as described before (7). Briefly, a wedge from the resected liver lobe was cut distant from the malignant lesion. Normal liver tissue was perfused (Masterflex pump, Novodirekt, Kehl am Main, Germany) at 37˚C with HBSS without Ca^{2+}/Mg^{2+} containing 100 μg/ml gentamycin sulfate and 20 mM HEPES, followed by a perfusion with 0.5 mM EGTA in HBSS without Ca^{2+}/Mg^{2+} under recirculation for 15-25 min. Subsequently, the liver tissue was perfused with 0.075% collagenase IV (Sigma) and 5 mM $CaCl_2$ in HBSS for 10-30 min. Liver cells were gently dispersed in cold HBSS with 20 mM HEPES-buffer and 100 μg/ml gentamycin sulfate. Hepatocytes were purified by triple centrifugation in DMEM/Ham's F12 medium (5 min, 28 x g). Cell vitality was estimated with trypan dye exclusion and only cells with a vitality >80% were utilized. Hepatocytes were seeded at a density of $1.8x10^5$ viable cells per cm^2 onto a collagen I-coated 6-well plate (Biocoat® BD Falcon, Heidelberg, Germany) in DMEM/Ham's medium containing 5% human serum, 10 ng/ml EGF, 100 μg/ml gentamycin sulfate, 20 mM HEPES.

Cell culture hypoxia and reoxygenation pattern. Four independent experiments with hepatocytes from different donors were performed. Hypoxia was induced by pregassing incubation medium with 100% N_2 for 30 min. Hepatocytes were then incubated in pregassed culture medium and sustained with 100% N_2 for 6 h at 37.0˚C and reoxygenated for 24 h. Cells were dislodged using accutase (PAA, Cölbe, Germany), centrifuged at 300 x g for 10 min. Cell pellets were snap-frozen in liquid nitrogen and stored at -80˚C.

LDH cytotoxicity assay. Cytotoxic cell death can be evaluated by quantification of plasma membrane leakage of LDH (8). Hepatocyte supernatant was collected and centrifuged at 170 x g for 10 min. Supernatant (100 μl) was transferred into a 96-well microtiter plate and 100 μl of reaction mixture (Cytotoxicity Detection Kit, Roche Diagnostics, Germany) was added and incubated for 30 min. The absorbance was read at 490 nm (Ceres UV 900C, Biotek Instruments Inc.,VT, USA).

Oxygen measurements. Oxygen concentration was measured in the culture medium at the beginning and at the end of each experiment (under N_2 atmosphere or under normoxic culture conditions). Oxygen content of the medium was reduced from 150 to 13.5 mmHg as measured by a portable O_2 analyzer (OxyScan graphic, UMS, Meinigen, Germany) according to the manufacturer's instructions.

Two dimensional gel electrophoresis. The 2-DE was performed with Zoom® IPGRunner™ (Invitrogen, CA, USA). In summary, $12x10^6$ hepatocytes were lysed in Zoom 2D protein solubilizer. Protein content was determined using Coomassie (Pierce, IL, USA). Zoom® strips pH 3-10 (Invitrogen) were rehydrated in 155 μl rehydrating buffer (8 M Urea; 2% CHAPS; 0.5% Carrier Ampholytes) and pooled protein samples (20 μg each). Pooled protein samples were composed by adding equal protein amounts from cell cultures of four independent cell-isolation and hypoxia experiments. The strips were subjected to isoelectric focusing according to manufacturer's instructions. For the 2nd dimension, NuPAGE® Novex 4-12% bis-tris gels (Invitrogen) were used. 2-DE runs were repeated three times for each pooled sample. Gels were stained with Sypro® Ruby stain (Molecular Probes, Leiden, The Netherlands) according to manufacturer's instructions. Proteins were detected using a Typhoon laser scanner (GE Healthcare, Munich, Germany). The quantification and comparisons of spot abundance was done after gel scanning as described before (3). Digital images were processed using the image software Phoretix 2D (Nonlinear Dynamics, Amersham Biosciences). Protein levels were compared among gels after normalization (total spot intensity). For the determination of significance ($p<0.05$) the Mann-Whitney test was applied.

Silver staining and spot excision. Before manual spot picking, gels were stained with Silver Stain Plus (Bio-Rad laboratories, CA, USA) according to manufacturer's instructions. Protein spots of interest were excised and stored at -80˚C.

Western blot analysis. Hepatocyte lysates (20 μg) were separated on a 7, 10 or 12% SDS-PAGE and transferred onto a PVDF membrane. Nonspecific binding was blocked by 10% dry non-fat milk in Tris-buffered saline for 1 h at RT and incubated with primary antibodies, anti-PCK1 1:1,000 (rabbit polyclonal, Genway Biotech Inc, San Diego, CA); anti-peroxiredoxin, 1:2,000 (rabbit polyclonal, Abcam, Cambridge, UK); anti-UGP2, 1:500 (mouse monoclonal, Abnova Corporation, Taipei, Taiwan); anti-FBP1, 1:1,000 (mouse polyclonal, Abnova Corporation); anti-CPS1, 1:200 (goat polyclonal); anti-GRP 78, 1:200 (rabbit polyclonal); anti-PGK1, 1:200 (goat polyclonal, all three Santa Cruz Biotechnology Inc., Heidelberg, Germany) diluted with 0.5% Tween and 0.5% BSA in Tris-buffered saline. Membranes were washed 3x with TBS-tween and incubated with secondary antibodies, goat anti-mouse IgG-HRP, 1:5,000 (Millipore, CA, USA); goat anti-rabbit IgG-HRP, 1:5,000 (Millipore); bovine anti goat IgG-HRP, 1:5,000 (Santa Cruz Biotechnology Inc.) diluted with 0.5% Tween and 0.5% BSA in Tris-buffered saline. Protein visualization was carried out using ECL™ Western blot detection reagent (GE Healthcare, Munich, Germany). To confirm equal loading, mouse monoclonal ß-Actin, 1:1,000 (Sigma Immnunochemicals, St. Louis, MO, USA) was used. Reprobing of all membranes was conducted using Western blot recycling kit (Alpha Diagnostic, TX, USA) according to the manufacturer's instructions.

MS analysis and bioinformatics. After manual gel el pieces underwent in-gel digestion (9) which was adapted for use on a Microlab Star digestion robot (Bonaduz, Switzerland) (10). Samples were reduced, alkylated, and digested using trypsin (Sigma Aldrich, Saint Louis, MO). The extracts were dried and stored at -20˚C until analysis. MALDI-TOF-MS and MALDI TOF/TOF MS/MS experiments were performed on a 4800 MALDI TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA). Samples were dissolved in 5 μl of water/acetonitrile/TFA (29.5/70/0.5, v/v/v). α-cyano-4-hydroxycinnamic acid (3 mg/ml) (Bruker Daltonics Inc., Manning Park Billerica, MA) in water/acetontrile/TFA (29.5/70/0.5, v/v/v) was used as matrix. Analyte and matrix were spotted consecutively in a 1:1 ratio and dried under ambient conditions. The dried sample was washed with ice-cold 5% formic acid to reduce salt contamination prior to analysis. Spectra were externally calibrated with a Sequazyme Peptide Mass Standards kit (Applied Biosystems). All spectra were processed using 4,000 peak explorer (noise filtering with correlation factor, 0.7; and advanced baseline correction with peak width, 32; flexibility, 0.5; degree, 0.1). Combined peaklists of each analysed sample were generated by peaks to mascot (Applied Biosystems) using deisotoped peaks with a signal to noise ratio of at least 15 for MALDI-TOF and 4 for MALDI-TOF/TOF data. Proteins were identified by Mascot (www.matrixscience.com, Matrix Science, Boston, MA) (peptide mass tolerance, 60 ppm; fragment mass tolerance, ±0.5 Da; maximum missed cleavages, 1) using the online NCBInr database (4872416 sequences; 1684850502 residues). Proteins with a score ≥79 were considered significant ($p<0.05$).

Results

Patient selection and demographic data. The patient samples for hepatocyte harvesting and isolation were selected according to their clinical history and intraoperative findings. Hepatocyte vitality was measured by trypan blue exclusion (78%, ±3.2%). Patient 1 (female, 55 years), patient 2 (female, 66 years) and patient 3 (female, 52 years) were resected for colorectal liver metastasis. Patient 4 (male, 64 years) underwent liver resection for liver cell adenoma.

LDH-release and oxygen levels. In order to analyze the proportion of cells with membrane damage and leakage as a consequence of cell death, an LDH cytotoxicity assay was performed (8). To ensure a valid control for the HR group it was relevant to exclude differing proportions of damaged cells in the two culture groups in order to rule out artifacts from proteome changes which are not due to regulation of living cells. The measurement of cytotoxicity 24 h after hypoxia and reoxygenation (HR) or normoxic (N) cultivation did not differ significantly between the groups (N, 21±13% vs. HR, 27±15%, n.s.). Additionally oxygen levels under HR conditions were monitored and maintained between 13.5-15% under N_2-athmosphere. In contrast, control oxygen values lay between 140 and 150%.

Protein abundance differences and protein identification. Using 2-DE, 401±40 distinct spots in the N group and 351±17 spots in the HR group were detected. A total of

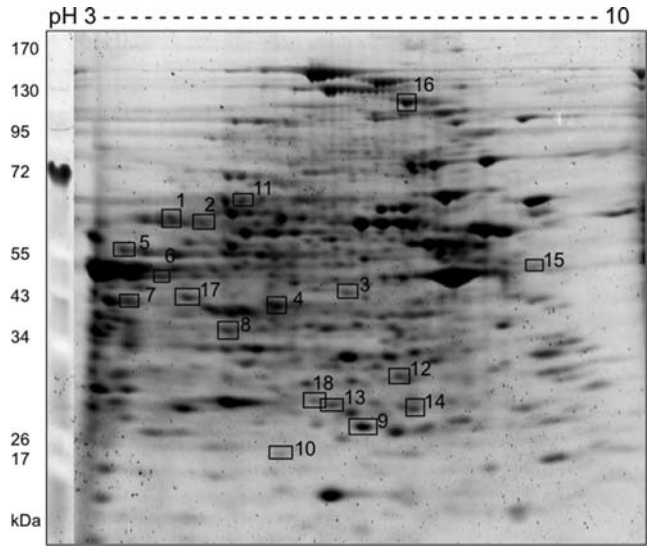

Figure 1. The 2D reference-gel (first dimension pH 3-10, second dimension 4-12% bis-tris gel) of pooled extracts from human hepatocytes originating from four patients and subsequent experiments are shown. The identified spots with significant abundance differences between the control group and after a sequence of hypoxia and reoxygenation are highlighted and numbered according to the text and Table I. Molecular weight markers are labeled on the left side of the gel.

509 spots were present in the reference gel allowing differential spot analysis of 262 spots (51%) present in all gels. Of these, a total of 106 spots (21%) displayed a >1.7-fold intergroup difference which was significant (Mann-Whitney) in 33 spots (6.5%) and qualified them for further identification by MALDI-TOF-MS. Out of this group, 18 spots (3.5%) (Fig. 1) were successfully unequivocally identified by mass spectrometry (Table I).

The identified proteins can be classified according to their functional properties. One protein involved in glycogenesis [UTP-glucose-1-phosphate uridylyltransferase (UGP2)] was present in two different spots (#314 and #328), both displaying reduced levels in the HR group compared to control (normoxic cells).

The pathway of gluconeogenesis was represented by phosphoenolpyruvate carboxykinase (PCK) (#467) and up-regulated in HR. Fructose-1,6-bisphosphatase (FBP) (#481) was down-regulated in HR.

Three proteins involved in glycolysis were uniformly down-regulated in the HR group, phosphoglycerate kinase 1 (PGK1) (#376), fructose 1,6-bisphosphate aldolase (ALDOA) (#427), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#470).

A set of three peroxisomal proteins was also identified. Thiosulfat-sulfurtransferase (TST) (#562) was found to be down-regulated, while α-class glutathione S-transferase (GST) (#655) and hydroxyacid oxidase 1 (HAO1) (#680) were both up-regulated in the HR group.

Four enzymes and one additional protein were identified that are involved in cellular oxidative and stress protection. Catalase isoform 2 (CAT) (#281) and peroxiredoxin II (PRDX2) (#636) were both up-regulated while thioredoxin peroxidase (PRDX4) (#600) and peroxiredoxin III (PRDX3)

Table I. The significantly altered 2D-spots with corresponding identified proteins.

Running number of identified proteins	Spot number	Protein name	Symbol	Protein function	Mass	M-score	Fold difference
Glycogenesis							
1	314	UTP-glucose-1-phosphate uridylyl-transferase	UGP2	Catalytic activity: UTP + α-D-glucose 1-phosphate = diphosphate + UDP-glucose	57,101	222	-3.8
2	328	UDP-glucose pyro-phosphorylase	UGP2	same as 314	49,005	179	-2.8
Gluconeogenesis							
3	467	Phosphoenolpyruvate carboxykinase	PCK	Catalytic activity: GTP + oxaloacetate = GDP + phosphoenolpyruvate + CO_2 (reversible rate-controlling step of gluconeogenesis)	71,447	88	+3.9
4	481	Fructose-1,6-bisphosphatase	FBP	Rate limiting enzyme in gluconeogenesis: Catalytic activity: D-fructose 1,6-bisphosphate + H_2O = D-fructose 6-phosphate = phosphate	37,190	117	-10.4
Glycolysis							
5	376	Phosphoglycerate kinase 1	PGK1	Catalytic activity: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate	41,773	140	-3.0
6	427	Fructose 1,6-bisphosphate aldolase	ALDOA	Catalytic activity: D-fructose 1,6-bisphosphate = glycerone phosphate + D-glyceraldehyde 3-phosphate	39,830	132	-5.0
7	470	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Catalytic activity: D-glyceraldehyde 3-phosphate + phosphate + NAD^+ = 3-phospho-D-glyceroyl phosphate + NADH	36,202	120	-2.6
Peroxisomal proteins							
8	526	Thiosulfat-sulfurtransferase	TST	Converts cyanide (CN-) to thiocyanate (SCN-) Catalytic activity: Thiosulfate + cyanide <=> sulfite + thiocyanate)	33,636	132	-2.9
9	655	α-class glutathione S-transferase	GST	Catalytic activity: RX + glutathione <=> HX + R-S-glutathione (conjugation of reduced glutathione via the sulfhydryl group to electrophilic centres on potentially toxic substrates)	19,624	166	+1.7
10	680	Hydroxyacid oxidase 1	HAO1	2-hydroxyacid oxidase most active on glycolate, also active on 2-hydroxy fatty acids, Catalytic activity: (S)-2-hydroxy acid + O_2 = 2-oxo acid + H_2O_2	27,405	118	+3.6
Oxidative and cellular stress protection							
11	281	Catalase isoform 2	CAT	Catalase-peroxidase: 2 H_2O_2 = O_2 + 2 H_2O; donor + H_2O_2 = oxidized donor + 2 H_2O	60,133	244	+2.2

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Continued.

Running number of identified proteins	Spot number	Protein name	Symbol	Protein function	Mass	M-score	Fold difference
12	600	Thioredoxin peroxidase	PRDX4	Ribonucleotide reduction: thioredoxin is oxidized from a dithiol to a disulfide, Catalytic activity: 2 R'-SH + ROOH = R'-S-S-R' + H_2O + ROH	30,749	114	-2.1
13	630	Peroxiredoxin III	PRDX3	Catalytic activity: 2 R'-SH + ROOH <=> R'-S-S-R' + H_2O + ROH	11,158	291	-2.1
14	636	Peroxiredoxin II	PRDX2	Catalytic activity: 2 R'-SH + ROOH = R'-S-S-R' + H_2O + ROH	16,165	149	+2.6
15	430	Heat shock 70 kDa protein 5 (HSP70)	HSPA5	Chaperone, cellular stress response protein: protein folding, -assembly, -degradation	51,197	172	+2.1
Other proteins							
16	140	Carbamoyl phosphate synthetase I	CPS1	Urea cycle, arginine synthesis, Catalytic activity: 2 ATP + NH_3 + CO_2 + H_2O = 2 ADP + phosphate + carbamoyl phosphate	165,975	371	+3.2
17	466	Annexin A2	ANXA2	Phospholipid binding protein	38,808	326	-2.7
18	624	Mitochondrial F1-ATP synthase, α subunit 1	ATP5A1	ATP generation	55,313	306	+1.9

Spot number, assigned number in 2D experiment; mass, molecular protein mass (Dalton); M-score, Mowse score (>79, $p<0.05$); fold difference, fold densitometric abundance difference of spots from hypoxia vs. control samples.

(#630) were down-regulated in the HR group. Heat shock 70 kDa protein 5 (HSPA5) (#430) was up-regulated in the HR group.

The mitochondrial F1-ATP synthase, α subunit 1 (ATP5A1) (#624), which is involved in cellular energy metabolism, was found to be up-regulated after HR. Carbamoyl phosphate synthetase I (CPS1) (#140), a member of the urea cycle, was up-regulated in the HR group, while annexin A2 (ANXA2) (#466) was down-regulated after HR treatment.

To confirm the observed 2-DE changes of the identified proteins, Western blot analysis was done with a selection of commercially available antibodies for each HR experiment separately. Due to limited antibody availability only seven of the identified proteins could be analyzed with this immunological assay. UGP2, PCK, PGK1 and PRDX2 showed similar changes in their levels as was seen in the 2-DE experiments (Fig. 2, right panel). In contrast FBP1, CPSI, and HSP70 did not display relevant changes in the Western blot (data not shown).

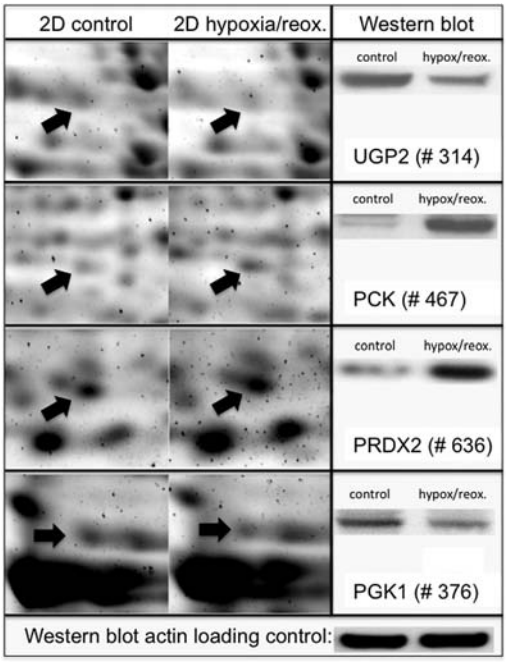

Figure 2. 2D page differences (left panels) andWestern blot analysis (right panels) of pooled hepatocyte lysates under control conditions vs. hypoxia and reoxygenation for four different spots and the corresponding proteins (UGP2, PCK, PGK1, PRDX2) are depicted. Results displayed are representative for the experiments performed. Western blot for actin served as loading control.

Discussion

Hypoxia followed by reoxygenation is a pathophysiological stimulus under various clinical conditions. The degree of the adaptive responses depends on the duration of hypoxia. Short-term reduction of O_2 supply is followed by the

modification of existing proteins through phosphorylation or other post-translational changes (11). When low O_2 concentration is sustained longer adaptive alterations of gene expression take place and finally chronic hypoxia results in cell death when the adaptive mechanisms are exhausted (12). Short-term hypoxia can prime cells or organs to increase their tolerance to hypoxia (13,14) for example through down-regulation of energy demanding pathways to reduce ATP requirements (15-17). Another compensatory factor is the up-regulation of protective components against reactive oxygen species generated during reoxygenation.

Since hypoxia represents a major challenge for cells, subsequent changes of gene expression, protein degradation, and other post-translational modifications should result in alteration of the proteome (18). To elucidate the global protective responses to HR in human primary hepatocytes we submitted their cultures to a sequence of hypoxia and reoxygenation. In contrast to immortalized cell lines the use of human primary hepatocytes in our analysis allows to imitate more accurately physiologic processes present *in vivo*. Proteome analysis of immortalized murine hepatoma cells revealed substantial differences to their primary counterparts (19). Following hypoxia the cellular proteome was analyzed after 24 h of reoxygenation in a steady state of oxidative cellular metabolism. These steady state conditions support the detection of prolonged proteome alterations possibly relevant for long term hepatocyte function without short-term artifacts due to cell death.

Cellular integrity and membrane damage was not significantly altered through our experimental set up as monitored by LDH release. The hepatocytes were capable of sustaining 6 h of hypoxia with O_2-values ~15 mmHg and reoxygenation without increase in LDH release. The critical level of 5 mmHg O_2 which would completely inhibit mitochondrial respiration (20,21) was always exceeded.

In this study proteome changes in three major functional protein groups, namely glucose metabolism, oxidative and cell stress protection as well as peroxisomal function were identified after the sequence of HR in human hepatocyte cultures.

Glucose metabolism. Our results illustrate that glucose metabolism remains affected even 24 h after HR. For example abundance of UGP2, involved in glycogenesis, was below control even 24 h after reoxygenation. This suggests that the cells still had not been able to regain their original energy status. UGP2 has also been shown to indirectly reduce the rate of glucuronidation under hypoxia by limiting the required substrates UTP and glucose 1-phosphate (22). The impairment of glucuronidation after LTX(23) might therefore partly be due to a prolonged down-regulation of UGP2.

The key enzyme of gluconeogenesis, FBP, was also identified. Liver net gluconeogenic flux is terminated during anoxia (24) and increasing AMP levels under anoxia act as an inhibitor of FBP (25). Although the 2-DE results indicated down-regulation of FBP, the Western blotting results showed no changes of FBP after HR. It is likely that regulation of FBP is not only influenced by changes of AMP levels but also involves post-translational changes causing a partial shift of the FBP spot in the 2D gels.

Glycolysis involves the identified enzymes PGK1, ALDOA, and GAPDH, which have all been linked to hypoxia dependent adaptation. PGK1 triggers HIF-1α-dependent gene transcription in response to hypoxia (26,27). ALDOA is up-regulated under hypoxia in astrocytes (28). GAPDH can form a complex with a ubiquitin ligase which initiates degradation of nuclear proteins during apoptosis (29). GAPDH also participates in the formation of the OCA-S transcriptional coactivator complex connecting the cellular metabolic state to gene transcription (30).

The reduced levels of the described glycolytic enzymes in our experiments after HR might reflect a compensatory metabolic state, which suppresses glycolysis under normoxic conditions after hypoxia to enhance restoration of glycogen and glucose levels.

Peroxisomal detoxification. The levels of three peroxisomal enzymes, TST, GST and HAO1, were altered due to HR. TST, a sulphur transferase, has a higher turnover rate under hypoxia and detoxifies cyanide (31). Our cells had a reduced abundance of TST after HR. GST, increased after HR, catalyses the addition of glutathione to different electrophiles resulting in protection against toxic substrates (32). An increase of GST activity under hypoxia was found in endothelial cells, medaka brain tissue (33), and in the frog *Rana pipiens* (34). Increase of GST activity is also required for hypoxia-dependent changes of the intracellular redox potential (35). Taken together, these findings stand in line with our observation of an anoxia-responsive regulation of GST.

HAO1, a liver specific oxidase (36), was also up-regulated after HR. Oxidase activity generates the reactive oxygen species hydrogen peroxide. Notably, in the context of HR peroxisomal oxidases are only minor contributors to the reactive oxygen species pool in hepatocytes especially in the presence of catalase activity (37).

Oxidative and cell stress protection. Four peroxiredoxins were differentially regulated after HR, CAT and PRDX2 (up-regulated), PRDX3 and -4 (down-regulated). Peroxidases (PRX) have a conserved Cys residue that undergoes oxidation and thiol-dependent reduction (38) during the removal of peroxides (39). Oxidation of PRX in the context of LTX is influenced by warm ischemia time (40) and PRX1 and -2 are induced during LTX resulting in protection against oxidant damage (41). Therefore, PRX induction might be an approach to protect transplant organs against IRI. This has already been shown in a murine IRI gene transfer model (42).

HSP70 has already been implicated in the cellular response to HR (43). Induction of HSP70 attenuated IRI in rat hearts (44) and during LTX (45). HSP70 expression does not occur during the stress period itself but during recovery after the noxae (18). This is supported by our finding of an HSP70 increase after reoxygenation. Again post-translational changes might explain the lack of Western blotting confirmation for the 2-DE changes in HSP70 abundance as phosphorylation of chaperones can result in a shift on the 2-DE page (46).

Proteins from other areas of cellular metabolism. Four proteins were identified originating from other metabolic contexts. CPS1 (urea cycle) influences the substrate L-arginine (47,48). L-arginine, the major source for NO, mainly originates from liver synthesis (49,50), and protects against IRI (51,52). It is intriguing to hypothesize that CPS1 up-regulation, as seen in

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ANXA2, also up-regulated after HR, is a phospholipid- and membrane binding protein. ANXA2 has been shown to protect neurons and glial cells against hypoxia injuries (53). Therefore, the observed ANXA2 up-regulation confirms previous findings and supports its role during hypoxic stress.

ATP5A1, a mitochondrial ATP-synthase up-regulated after HR, hydrolyzes ATP to counteract the collapse of the proton motive force under hypoxic conditions. Thus ATP consumption by ATP-synthase must be limited under anoxic conditions by inhibition of the enzyme (54,55). It was proposed that mitochondria are regulated at the level of the ATP-synthase *in vivo* (56). This regulation involves gene expression changes as shown after HR in astrocytes. Interestingly, hypoxia alone did not induce ATP-synthase gene expression but it occurred after reoxygenation (57).

The proteomic analysis of hepatocytes submitted to sequential hypoxia and reoxygenation revealed three major metabolic areas to be affected in response to the stimulus. Among these were glucose and glycogen metabolism (six proteins), the process of peroxisomal detoxification (three proteins) and mechanisms of oxidative and cellular stress protection (five proteins). Many of the proteins identified have already been implicated in the context of cellular adaptation mechanisms in response to HR. It appears that phylogenetically old protective mechanisms play a role in stabilizing hepatic cellular functions. Further studies are needed to elucidate the relevance of the observed enzyme and protein changes in the clinical setting. Some proteins might have diagnostic potential in the evaluation of liver graft quality or could serve to monitor therapeutic interventions such as glucose supply to potential organ donors to optimise the energy status of the organs to be harvested (58).

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