Oxidative Stress in Sepsis: Implications on Liver Protein Patterns and Analysis via Modified Proteomics Technology

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Introduction

Sepsis is the systemic response of the host organism to the invasion of microbial species and/or their toxins. The incidence of sepsis is steadily increasing, and, despite recent progress in intensive care research, mortality is still high, in particular when septic shock and multiple organ failure (MOF) develop [1, 2]. Diverse molecular mechanisms of inflammation and cellular damage have been implicated in the pathogenesis of septic shock and MOF [3, 4], including the excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Many cellular processes, such as inflammatory host defense and energy metabolism involve redox processes, which take place all over the cell comprising simple electron transfer reactions, radical processes as well as thiol/disulfide exchanges. To ensure proper function, the living cell has to monitor, control and maintain the intracellular redox balance. However, in septic shock an imbalance between ROS and antioxidant defense mechanisms occurs, resulting in oxidative stress [5, 6]. The reason for this imbalance is an overwhelming production of ROS and/or a deficit in antioxidant systems. The most important ROS/RNS are represented by the following candidates: superoxide anion ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), hydroxyl radical ($HO^{\bullet -}$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (see Table 1). Among the various ROS, $O_2^{\bullet -}$ plays a key role in the pathogenesis of hemodynamic instability and organ dysfunction during septic shock. $O_2^{-\bullet}$ is primarily produced by activated neutrophils and macrophages as part of the innate immune system [7, 8] and has been associated with the inflammatory response that accompanies tissue damage in septic shock [9]. Beside non-enzymatic antioxidants, e.g., vitamins C and E, bilirubin, reduced glutathione and albumin, superoxide dismutase (SOD), catalase and glutathione peroxidase are referred to as major *enzymatic* antioxidant systems. Under normal conditions, the formation of $O_2^{\bullet -}$ is kept under tight control by endogenous SOD enzymes. Despite their importance in innate immunity representing one important defense mechanism against invading pathogens [6], the overwhelming production of ROS threatens the integrity of various biomolecules including proteins [10], lipids as well as lipoproteins, and DNA [11] resulting in tissue damage, by lipid peroxidation of cell membranes, protein oxidation and DNA strand breaks. These pathomechanisms contribute to MOF during sepsis resulting in myocardial depression, hepatocellular dysfunction, endothelial dysfunction, and vascular catecholamine hyporesponsiveness.

It must be underscored that, beside the negative effects associated with oxidative stress, ROS exert several important and vital beneficial physiological cellular functions which have been demonstrated in different areas including intracellular sig-

Table 1. Reactive oxygen and nitrogen species (ROS/RNS): origin and metabolism

ROS/RNS molecule	Main Source	Enzymatic Defense System	Products
Superoxide ($O_2^{\bullet -}$)	Activated phagocytes Discharge of electrons from the electron transport chain Xanthine oxidase Flavoenzymes	Superoxide dismutase (SOD)	$H_2O_2 + O_2$ H_2O_2
Hydrogen peroxide (H_2O_2)	Product of SOD NADPH-oxidase (neutrophils) Glucose oxidase Xanthine oxidase	Glutathione peroxidase Catalases Peroxiredoxins (Prx)	$H_2O + GSSG$ $H_2O + O_2$ H_2O
Hydroxyl radical ($HO^{\bullet -}$)	From $O_2^{\bullet -}$ and H_2O_2 via transition metals (Fe or Cu)		
Nitric oxide (NO)	Nitric oxide synthases	Glutathione/TrxR	GSNO
Peroxynitrite ($ONOO^-$)	Product from $NO + O_2^{\bullet -}$ triggering DNA single strand breaks	Poly-(ADP-Ribose) Polymerase (PARP)	

SOD: superoxide dismutase; GSSG: oxidized glutathione; GSNO: S-nitrosoglutathione

naling and redox regulation [12, 13]. First, ROS represent a defense mechanism against invading organisms by activated phagocytes [6]; ROS are produced by the NADPH oxidase complex in this system. Second, ROS can directly affect the conformation and/or activities of all sulfhydryl-containing molecules, such as proteins or glutathione by oxidation of their thiol moiety [13]. For example, superoxide, hydrogen peroxide, and NO are well known regulators of transcription factor activities and other determinants of gene expression [14–16]. Several cytokines, growth factors, hormones and neurotransmitters use ROS as secondary messengers in the intracellular signal transduction [17]. Well-known examples of redox-sensitive transcription factors are nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) [15, 18], the nuclear factor-E2 related factor 2 (NrF2) pathway targeting the antioxidant element [19], or the ROS mediated sensing of hypoxia [20]. The mechanisms for altered transcription factor control could be either via decreased binding to promotor regions via oxidative damage to the DNA or more direct by redox regulation of transcription factor activation [21] and/or altered DNA-binding due to redox-induced modification of the transcription factor protein [22]. Third, in addition to their physiologic beneficial effects, ROS are, due to their high reactivity, prone to cause damage being, thereby, also potentially toxic, mutagenic or carcinogenic. Thus, the targets for ROS/RNS damage include all major groups of biomolecules as already mentioned above: proteins, lipids, DNA.

As already mentioned, there are several cellular antioxidant enzymes, such as (the well characterized) SOD, as well as complex systems such as the cysteine-based redox regulation of the glutathione and thioredoxin pathways [22]. The SOD enzymes include the copper/zinc enzymes present in the cytosol (SOD1) or extracel-

lular surfaces (SOD3), and the manganese enzyme in the mitochondria (SOD2). In disease states, the production of $O_2^{-\bullet}$ is increased at a rate that overwhelms the capacity of the endogenous SOD defense system, resulting in $O_2^{-\bullet}$-mediated damage. The proinflammatory properties of $O_2^{-\bullet}$ include endothelial cell damage and increased microvascular permeability [23, 24], formation of chemotactic factors, e.g., leukotriene B_4 [25], recruitment of neutrophils at sites of inflammation [26], lipid peroxidation and oxidation, DNA single strand damage [27], release of cytokines, e.g., tumor necrosis factor (TNF)-α and interleukin (IL)-1β [28, 29], and formation of $ONOO^-$, a potent cytotoxic and pro-inflammatory molecule triggering DNA single strand breaks [30, 31].

Regarding the glutathione- and thioredoxin-reduction pathways, it has become clear that there are two parallel, interdependent enzymatic systems. On the one hand, glutathione as a reducing substrate seems to be more effective in reducing small disulfide molecules and in reacting directly with ROS, whereas, on the other hand, thioredoxin is more effective in reducing the exposed disulfides of proteins. Thus, the thioredoxin system can also be seen as an antioxidant defense/repair system for (accidentally) oxidized cysteine proteins [22].

Reduced glutathione is among the most important intracellular antioxidant within human cells. It exists in equilibrium with its disulfide form (GSSG), and the ratio of glutathione to GSSG could be used as an indicator of the redox status of the cell. Several important human antioxidant-defense systems are based around glutathione, e.g. glutathione peroxidase as a major cellular reducer of hydrogen peroxide (together with catalase and peroxiredoxin) [22]. Another important element of the defense system is formed by chaperones or heat shock proteins (HSP) [32]. Oxidized proteins tend to change their tertiary structure and when the oxidation is reversed they have to be refolded by chaperones to gain their optimal structure. Chaperones like HSP27 and HSP70 have an anti-apoptotic signaling effect. Also, the protein refolding requires ATP, increasing the energy requirement of the defense system, which also consume energy for the regeneration of reducing equivalents like NADPH. The latter is provided by pentose phosphate shunt activity or mitochondrial oxidation. Break down of the energy supply leads to apoptosis, which is reflected in a tight coupling between these two processes [33].

Under sepsis, various processes, triggered by ROS/NOS contribute to oxidative stress. Their interrelation is outlined in Figure 1. It appears that stimulated oxidative processes form one key element in the cascade of deleterious processes. Therefore, as a causative therapy, the antioxidant system should be supported. This concept has been the subject of intensive discussion [34, 35], but the results at best have been rather equivocal, to some extent certainly due to the 'friend and foe' properties of antioxidant supplementation, which was elegantly characterized as the "antioxidant paradox" [36]. Thus, the multiple interrelated processes make it difficult to assess where, when, and in which dose the supporting agent should be delivered. What is missing so far is an integrated approach that allows characterization of the state or activity of the various processes involved in oxidative stress and its defense responses. cDNA-micro array measurements [37, 38] provide such 'holistic' information about gene activation, as reflected in the mRNA levels. Microarray data allow the definition of expression patterns for specific disease states and, hence, could be used as a tool to classify sepsis or oxidative stress. Furthermore, they may reflect the state of the signaling system, but they cannot be used to infer on the activity of the proteins or enzymes they encode, because many other steps, in addition to transcription, may affect the activity of an enzyme. Hence, complementary information would be useful

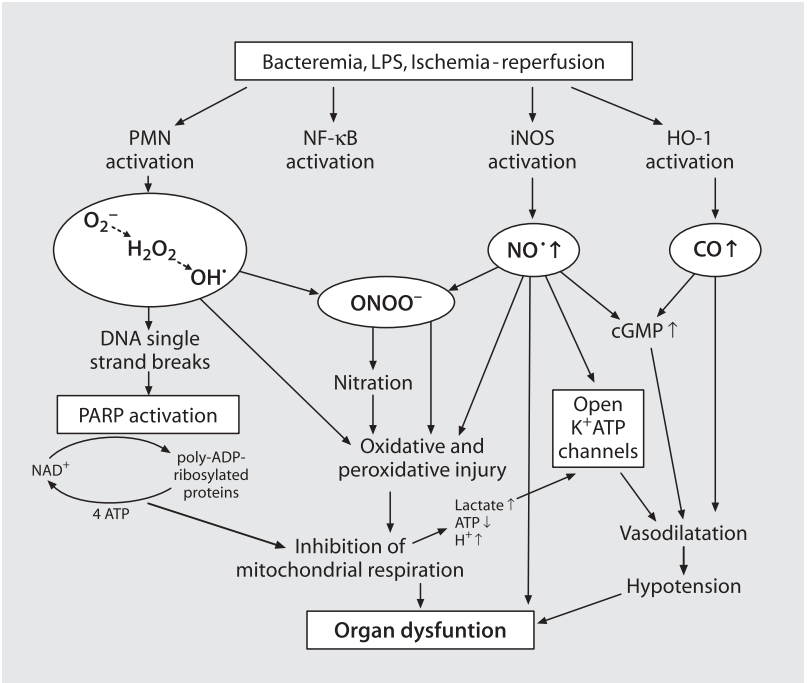

Fig. 1. Schematic representation of oxidative stress processes contributing to organ disfunction during sepsis. CO: cardiac output; NO: nitric oxide; LPS: lipopolysaccharide; iNOS: inducible NO synthase; HO: heme oxygenase. From [50] with permission

to describe the functional status of the proteins involved in the defense system, which in turn leads to the analysis of the cell proteome.

Outline of the Defense System

The major event of oxidative stress is the generation of H_2O_2, which in turn oxidizes proteins and other cellular components. As the enzymatic defense system consists of proteins, damage to the defense proteins is particularly harmful, as it may directly lead to cell death. We focus here on specific cellular strategies to repair or avoid damage and prevent oxidative inactivation of proteins, its reversibility, to remove irreversibly damaged proteins and, if necessary, to replace them by *de novo* synthesized ones. Figure 2 depicts some elements of the defense system.

The oxidation can be reversed at the expense of glutathione or NADPH consumption, which have to be regenerated via mitochondrial oxidation of glucose or the activity of the pentose phosphate shunt. The efficiency of this regeneration is monitored via the redox state of various proteins like thioredoxin or signaling proteins involved in the NF-κB or NrF2 pathway. Via this signaling the transcription of enzymes for the defence system is initiated, increasing the defence capacity. Oxidation or overoxidation of the Cys-SH groups or other amino acid residues changes the tertiary structure of the protein, which in turn evokes a misfolded protein sig-

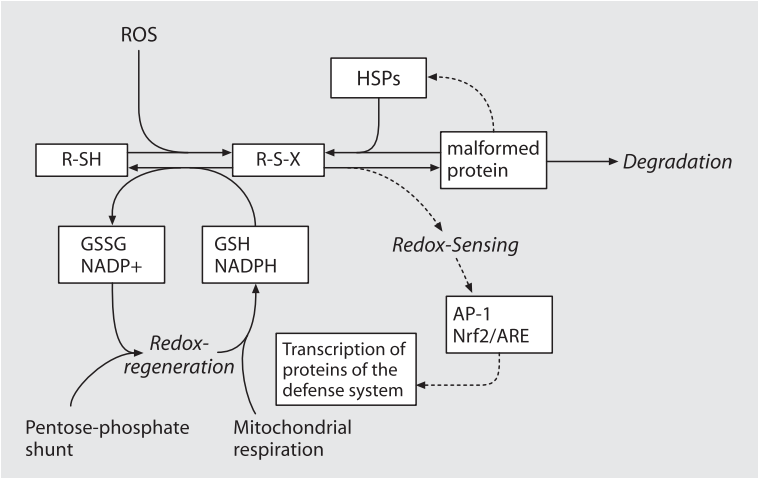

Fig. 2. Structure of a cellular defense system focusing on oxidative protein damage, protein repair, degradation and signaling pathways, regulating their interplay. Solid lines indicate flow of material or chemical reactions. Broken lines indicate stimulatory signaling effects. HSPs: Heat shock proteins; R-SH, R-SX: Proteins with free and oxidized thiol group of cysteine, respectively; ARE: anti-oxidant responsive element; NrF2: NF-E2 related factor 2; AP-1: activator protein-1

nal. This activates the chaperones and other components of the protein repair system. If the oxidation is irreversible, then the protein must be degraded.

Single elements of this defense system have been described in many cases; we are interested in the integration and coordination of the various defense processes in a clinically relevant sepsis model, and the chances to monitor them using protemic tools. The latter are geared to assess changes in the protein content of a cell or tissue.

Proteomic Tools

We are using functional and quantitative proteomic tools to monitor characteristic differences in the levels and turnover rates of selected proteins from control, sham-operated, and septic mice. A prominent tool is based on the separation of a protein mixture, derived from tissue or cell extract gel-electrophoresis, in two dimensions (2D): one for the molecular weight of the protein, and the second separating along the pKi-value of the protein. An individual protein appears as a single spot on the gel, and depending on the size of the gel and sampling conditions, more than 1000 different proteins can be separated. After 2D-separation the proteins in the acrylamide gel have to be chemically treated to become detectable. This can be done using organic dyes (coomassie brilliant blue), metal ion reduction (silver staining), fluorescence labels, or radioactive isotopes. The intensities of the detected spots reflect the protein content of the analyzed tissues or cells [39]. Changing the metabolic or stress conditions will alter the protein content. To quantify these changes, protein extracts obtained from a study and a reference sample can be tagged or labeled with different isotopes (e.g., I^{125} and I^{131}, attached to cysteines in the amino acid chain of the protein). This mixture is then separated on a single gel and the

various protein spots contain both labels (i.e., I^{125} and I^{131}). The relative proportion of these labels reflects the proportion of the specific protein in the study sample relative to the reference. The detection of the radioactive label is very sensitive and can be visualized. Changes in the protein profile can be detected including proteins that are present in low numbers of copies per tissue [40], which allows a 'differential' expression pattern to be characterized. As the protein expression is a function of signaling in the frame of regulation networks, it should provide the opportunity to assess the state of the signaling system, reflecting the activity of cytokines and other mediators, and changes thereof.

Differential expression figures, albeit very sensitive, do not provide identification of the protein spot. A common approach for identification, therefore, is based on mass spectrometry: the spot is excised from the gel and digested with a specific protease like trypsin. The protease always cuts the amino acid sequence at specific points (after Lys and Arg). With such a specific segmentation each protein yields a unique pattern of peptide fragments. The digest can be analyzed with Maldi-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry, which allows detection and identification of the peptides by their molecular weight. The resulting set of observed peptides is matched to a database that contains a large set of proteins and all their peptides, predicted by theoretical tryptic fragmentation from their amino acid sequence. This protein identification is termed peptide mass finger printing (PMF). PMF is usually applied to proteins separated by 2D-gel electrophoresis. As a very sensitive detection method, silver staining is used to visualize proteins on the 2D-gels. Together with a rough quantification of the amount of protein by virtue of silver staining intensity spot, this allows to quantify and identify hundreds of proteins of a cell or tissue extract. There are, however, some limits to this concept related to the capacity and separation power of the gel and the primary detection. Only spots that can be visualized by staining can be analyzed on the gel. The dynamic range of this method is about $1:10^3$, thus only proteins with a content larger than 0.1% of the most abundant proteins can be monitored. The dynamic range in living cells from structural proteins to signaling proteins, however, is in the range of $1:10^6$. Thus, only the abundant fraction of proteins can be monitored. Moreover, general gel electrophoresis procedures are confined to proteins with a pKi value between 3 and 10 and a molecular weight larger than 10 000 Dalton.

Based on these limitations, activation of the signaling proteins depicted in Figure 1 cannot be completely visualized with the 2D-PAGE Maldi-TOF platform. Hence, the focus generally moves onto abundant proteins or enzymes, which perform the real defense task and that are regulated by the signaling structure outlined before.

What are typical defense proteins?

- Cytosolic proteins like SOD1, catalase and glutathione-peroxidase which act as first scavengers for ROS molecules.
- Glyoxalase, aldehyde reductases and enolase, which detoxify small molecules (specifically aldehydes) generated by ROS activity.
- Proteins involved in the redox-metabolism like glutathione-S-transferase, thioredoxin and peroxiredoxin.
- Proteins stimulated by the 'malformed protein signal' via the heat shock factor (HSF)-pathway [32] or from the protein repair system, like HSP27, HSP70, HSP90, protein disulfide isomerase (PDI).

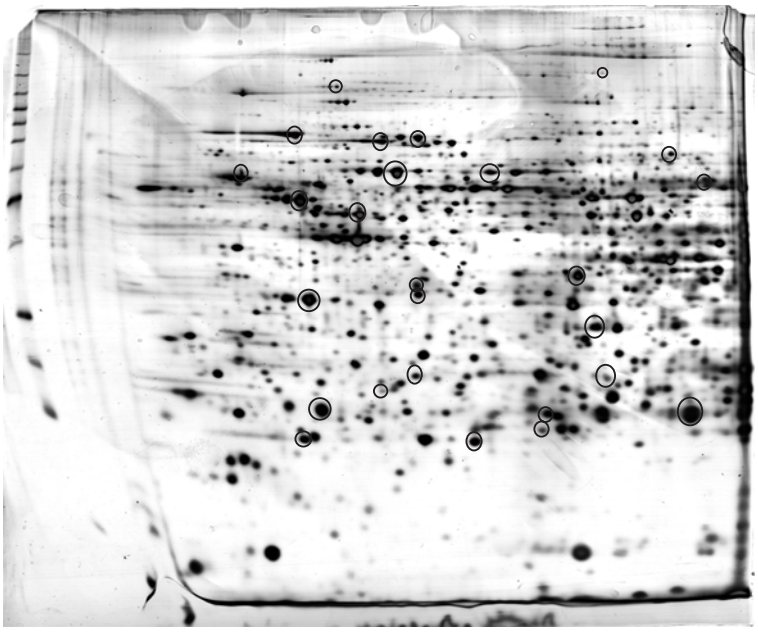

Fig. 3. SDS 2D-gel, containing proteins of a male mouse liver after surgical stress. Circled spots indicate proteins involved in the oxidative defense system, as identified by us

- Key enzymes of glycolyses and the pentose-phosphate-shunt, which provide ATP for the protein and DNA repair systems and the reducing equivalents NADH or NADPDH for the defense system.

All these proteins are part of the cellular defense system. Moreover, ROS species are generated by incomplete reduction of oxygen during cellular respiration even under resting, non-challenged conditions. They cause, to a low extent, the above-mentioned damage and, hence, a substantial constituent amount of the stress proteins is required. The same holds true for the protein repair system, which also functions as a quality control system for the protein synthesis machinery. Consequently most of these proteins are expressed at high, abundant rates even under non-stress conditions. Figure 3 shows a 2-D gel with defense proteins of the liver extract extracted from a mouse subjected to surgical stress. It turns out that defense proteins form a large, significant part of the gel-picture, and there is no need to resort to extreme stress conditions to visualize these proteins.

Dynamic Proteomics

Changing the oxidative stress level should induce changes in the expression profile of the proteins, and it is tempting to assign and trace back these expression changes to changes in cytokine activation and signaling pathways. In a model of chemically induced oxidative stress, Xiao et al. [41] exposed macrophage cells to

three different levels of diesel exhaust particles, and found a reduction in the glutathione/GSSG ratio as a function of the stress level as well as different protein expression patterns for these levels. From the gene regulation of various proteins they could assign the expression changes to changes in stimulatory pathways. For 'low' stress levels they found Nrf-2-driven changes targeting anti-oxidant response elements, for their 'medium' levels NF-κB/mitogen-activated protein kinase (MAPK)-driven changes that affect AP-1 target. The 'highest' level was associated with mitochondrial damage and apoptosis. Among the proteins identified were signaling proteins like p38, MAPK/ERK kinase kinase 1 or TNF-receptor. The latter, however, are only visible in isolated cells of the immune system and unlikely to be prominent in tissue extracts. The paper by Xiao et al. [41] clearly demonstrates both the strengths and limitations of the 2D-PAGE Maldi-TOF proteomics approach: The expression profile covering many proteins and its change, caused by altered conditions, can be monitored, and the amount of information available drastically exceeds that available using other protein-biology methods targeting specific enzymes based on antibodies or enzyme activity. The expression patterns shown, thus, would be sufficient to quantify an unknown stress level for a cell sample and to characterize the defense response. There are, however, limitations. For example, only a fraction of the enzymes involved in the antioxidant defense are listed. The above-mentioned defense and redox protein are missing despite the fact that their synthesis would be expected to be stimulated by the activation of the stress signaling pathways. These limitations arise from the detection based on spot size or intensity. First, the detection is only static, i.e., depicts protein content, but cannot separate between synthesis and breakdown, in other words, turnover rate. Second, the premise that the content of a protein is proportional to the size or staining intensity of a single spot, does not hold in each case: Defense and redox proteins tend to have a free Cys-SH group that can be reversibly oxidized or linked to glutathione, as shown for T-lymphocytes [42] and hepatocytes [43]. Free Cys-SH groups of the protein repair enzyme, PDI, oxidize or reduce the cysteine groups of other proteins, to form or break disulfide bridges that stabilize the 3D-structure of a protein, and, thereby, are susceptible to oxidation. Since sulfur can undergo different oxidation steps, irreversible overoxidation is also possible. In parallel, the residues of other amino acids can be oxidized or carbonylated [44]. All these steps shift the pKi value of the affected proteins toward more acidic values. As a consequence, some proteins appear in different spots on the gel and, in the extreme case, may depict a 'pearl chain' pattern, as seen in Figure 3. Furthermore, in some instances the protein chain is either cleaved *in vivo* by partial proteolysis to two smaller sub-chains, or the chain breaks during sample workup leading to two different protein spots with molecular weights much lower than expected. Taken together, the moiety of specific protein can be distributed over different spots. Some of these isoforms can be overoxidized and inactive, accumulating and designated for degradation [45, 46], and cannot be referred to as contributing to the content of the biologically active isoform. For example, for peroxiredoxin 2 and 3, Rabilloud et al. [45] demonstrated inactivation by irreversible oxidation of cysteine to cysteic acid. The oxidized form appears as a more acidic spot in the gel and was suggested as a marker for oxidative protein damage. Although there was a significant conversion from the active to the inactive, acidic form, the size of the spot, representing the active form of the protein, did not decrease significantly. In a subsequent study [47], it was shown, by using stable isotope labeling, that the active version of the protein was regenerated by *de novo* synthesis, whereas the oxidized

protein was not regenerated to the active form but degraded. The size or staining intensity of the active protein spot remained unchanged, as an increased synthesis was paralleled by increased irreversible conversion to an inactive state. In this specific case, spot size or measurable protein expression was not proportional to protein synthesis, violating the above-mentioned premise for profiling.

A similar situation was observed for the protein disulfide isomerase [48], a protein involved in protein folding/repair, located in the endoplasmic reticulum. Again, the loss due to protein oxidation was compensated by *de novo* synthesis. This paper also demonstrated that oxidized proteins are degraded by the proteasome system. The same group showed that oxidized proteins change their tertiary structure to unshielded hydrophobic amino acid residues [46], like large bulky aliphatic side chains or aromatic residues, and postulated that the altered hydrophobicity is the key signal for recognition by the proteasome for degradation. Oxidation-induced degradation by the proteasome has been shown for other proteins like SOD1 or mitochondrial aconitase, a key enzyme of the Krebs cycle [46].

Is oxidative protein damage a random event, affecting all proteins to a similar extent? In a study using irradiation as source for oxidative stress, Magi et al. [44] screened the cellular proteom for carbonylation and found a preferential damage for HSP next to structural proteins and some other proteins of the defense system. The preferential damage of HSP was explained with the tight contact these proteins have with other damaged proteins during protein repair, which might increase the likelihood of radical transfer from the damaged to the repair protein.

What does preferential oxidation and inactivation combined with increased degradation imply for an integrated defense system and its regulation as outlined in Figure 1? In this figure R-SH reflects any protein with a free Cys-SH group, including proteins of the defense system. Their inactivation by oxidation could lead to their degradation and trigger, via the pathways outlined there, the synthesis of new defense proteins. For PDI and peroxiredoxin, it was demonstrated that such a regulatory feedback loop could compensate for oxidative loss via signaling pathways that are not yet completely uncovered. It is also conceivable that any 'cross talk' between the different signaling methods may stimulate the expression of other proteins. This interplay cannot be detected based on monitoring the spot size/intensity alone, and consequently additional measurements for protein breakdown or synthesis are required. Using such a combined approach a detailed characterization of the defense state is possible. For example, under overwhelming toxic oxidative stress, we expect that the compensatory *de novo* synthesis will not compensate for the oxidative loss, which should be reflected in a reduced size of the spot for the active protein, increased turnover, and eventually increased size of the oxidative spot. Therefore, we recently developed an approach [49] to measure the fraction of a protein pool that is derived by *de novo* synthesis during the labeling phase (fractional synthesis rate), the proteins of which were separated and identified by 2D-gel electrophoresis and Maldi-TOF mass spectrometry. This approach is based on metabolic labeling with ^{13}C during constant infusion of uniformly labeled glucose and is sensitive enough to detect small fraction synthesis rates as low as 2% and changes thereof in the range of 0.5%. Table 2 lists the results for some proteins, which are related to the above mentioned defense system.

For a proof of principle, in a pilot study we compared the fractional synthesis rates of HSP between a septic and a sham operated condition. During sepsis we found a significant reduction in the synthesis of HSC70, a constitutive chaperone, virtually no change for HSP60 and HSP70, and a significant increase for PDI. This

Table 2. Fractional synthesis of individual proteins, participating in the antioxidant defense system, obtained from an unchallenged mouse liver

Protein	Fractional synthesis rate (± SEM)
Glutathione-S-transferase	2.8 (0.6)
Cellular glutathione peroxidase	0.7 –
Peroxiredoxin 2 (thioredoxin peroxidase 1)	5.2 (2)
Cu/Zn superoxide dismutase (SOD1)	0.9
Heat shock protein 60 (HSP60)	1.5 (0.5)
Heat shock cognate protein 70, heat shock 70 kD protein 8	15.1 (0.8)
Protein disulfide protein	8.6 (0.6)
Glucose regulated protein	9.5 (0.5)

encouraged an, at present still ongoing, detailed analysis of sepsis-induced changes in turnover and content for different defense proteins.

Conclusion

Taken together, proteins of the antioxidant defense system can be damaged by oxidative stress, and, in fact, there is evidence that they are even specifically susceptible. The oxidative loss of protein moiety is partially compensated by *de novo* synthesis. This compensatory mechanism complicates any attempt to relate mRNA profiles assessed by cDNA technology or protein expression profiles assessed by 2D-gel electrophoresis to the functionally active protein content. The dynamic cellular response with sepsis can only be revealed by disentangling the enormously complex response at the protein level. The only method able to deliver appropriate information is a proteomic platform based on differential and quantitative approaches, which is extended by synthesis or turnover measurements.

Our ultimate aim is to use this dynamic approach:

- to understand the complex interaction between the various elements of the defense system;
- to define a set of measurements necessary to characterize the various conditions of the system; and
- to develop a tool box to evaluate the efficacy of therapeutic measures intended to support the defense system.

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