This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Gene-specific regulation of hepatic selenoprotein expression by interleukin-6

J. Martitz\textsuperscript{a}, N.-P. Becker\textsuperscript{a}, K. Renko\textsuperscript{a}, M. Stoedter\textsuperscript{a}, S. Hybsier\textsuperscript{a}, and L. Schomburg\textsuperscript{a}

Sepsis is a severe inflammatory disease resulting in excessive production of pro-inflammatory cytokines including interleukin-6 (IL-6), causing oxidative stress, tissue damage and organ dysfunction. Health benefits have been observed upon selenium (Se) supplementation in severe sepsis. Selenium is incorporated into selenoproteins implicated in anti-oxidative defence, thyroid hormone metabolism and immune regulation. Selenium metabolism is controlled by hepatocytes synthesizing and secreting the Se transporter selenoprotein P (SePP). Circulating SePP declines in sepsis causing low serum Se levels. Dysregulation of the hepatic selenoenzyme deiodinases type 1 (DIO1) potentially contributes to the low T3 (thyroid hormone) syndrome observed in severe diseases. We hypothesized that IL-6 affects hepatic selenoprotein biosynthesis directly. Testing human hepatocytes in culture, IL-6 reduced SePP mRNA and secreted SePP concentrations in a dose-dependent manner. In parallel, expression of DIO1 declined on the mRNA, protein and enzyme activity level. Effects of IL-6 on glutathione peroxidase (GPX) expression were isozyme-specific; GPX1 remained unaffected, while transcript concentrations of GPX2 increased and of GPX4 decreased. This pattern of IL-6-dependent effects was mirrored in reporter gene experiments with SePP, DIO1, GPX1, and GPX2 promoter constructs pointing to direct transcriptional effects of IL-6. The redirection of hepatic selenoprotein biosynthesis by IL-6 may represent a central regulatory circuit responsible for decline of serum Se and low T3 concentrations in sepsis. Accordingly, therapeutic IL-6 targeting may be effective for improving Se and thyroid hormone status, adjuvant Se supplementation success and survival in sepsis.

Introduction

Severe sepsis and septic shock are the major causes of death in the intensive care units.\textsuperscript{1} Sepsis is defined as a systemic inflammatory reaction towards an infection, which is associated with an excessive release of pro-inflammatory cytokines and chemokines by immune cells such as lymphocytes and macrophages. Liver takes central stage in the defense against invading bacteria, and hepatic macrophages, i.e., Kupffer cells, account for circa one third of the nonparenchymal liver cells in normal mice.\textsuperscript{2} Upon infection, activated hepatocytes, stellate cells, cholangiocytes and endothelial cells amplify the pro-inflammatory response by secretion of interleukin-1\textbeta, tumor necrosis-factor \alpha and interleukin-6 (IL-6). The local cytokine and chemokine milieu finally controls hepatocyte activity, cell death or survival, immune cell infiltration, angiogenesis and other hepatic responses.\textsuperscript{3} Among the plethora of diffusible signals, IL-6 acts as the main stimulator of the biosynthesis of hepatic acute phase proteins (APPs).\textsuperscript{4} APPs participate in antimicrobial effects, e.g., the complement factors, ferritin or C-reactive protein. In the context of trace element metabolism, the copper (Cu) transporter ceruloplasmin (CP) is an established up-regulated APP and direct IL-6 target gene.\textsuperscript{5} Among the selenium (Se)-dependent proteins, selenoprotein S (SELS) becomes induced by inflammation\textsuperscript{6} and under ER-stress.\textsuperscript{7} SELS is an important component of the quality control machinery for newly synthesized proteins operative in the endoplasmic reticulum (ER).\textsuperscript{8} Genetic association studies and in vitro experiments have indicated that single nucleotide polymorphisms (SNP) in SELS causing reduced SELS expression are associated with increased pro-inflammatory cytokine release in humans.\textsuperscript{9} These findings highlight a direct interaction of inflammation, ER stress and APP biosynthesis with the expression of selenoproteins.
The dependence of ER functioning and APP biosynthesis on a Se-dependent protein seems counter-intuitive at first sight, as Se is known as a negative acute phase reactant, and blood Se concentrations and circulating levels of the Se transport protein selenoprotein P (SePP) decline in sepsis.10, 11 The negative acute phase-response of serum Se concentrations has been verified in a murine model of septic shock, where endotoxin (LPS) exposure caused reduced SePP biosynthesis and secretion.12 These findings pointed to cytokine-dependent regulation of hepatic selenoprotein biosynthesis under inflammatory conditions. As IL-6 constitutes a central component of the inflammatory response and some studies observed an inverse association between serum Se and IL-6 concentrations13, we hypothesized that IL-6 affects hepatic selenoprotein expression by direct mechanisms. We found an IL-6-dependent shift in the relative mRNA and protein concentrations of hepatic selenoproteins with increased levels of intracellular GPX activity at the expense of SePP biosynthesis and secretion, which likely contributes to the declining serum Se concentrations and deranged thyroid hormone pattern observed in critical illness.

Experimental

Cell culture

Human hepatoma HepG2 and Hep3B cells were grown at 37°C and 5% CO₂ in DMEM/Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS). Cells were seeded and treated with sodium selenite (100 nM Na₂SeO₃) and/or IL-6 (10, 20, 50, 100 or 200 ng/ml IL-6 for 24 or 48 h) (Biomol GmbH, Hamburg, Germany) in FBS- and phenol-red free medium. At the end of the incubation periods, supernatants were collected, cells were harvested, immediately frozen or homogenized, and expression of selenoproteins was analyzed.

Plasmids and luciferase reporter gene assays

Core promoter regions of ~1 kb DNA upstream of the transcription start sites were amplified from human DNA and subcloned into the luciferase reporter plasmid pGL4.10[Luc2] (Promega, Mannheim, Germany). Reporter gene assays were conducted essentially as described.14 For each experiment, 20,000 cells/well were seeded into 96-well plates and transfected with 95 ng reporter and 5 ng pSEAP-control vector (Promega) per well using linear polyethyleneimine (PEI, 25 kDa) (Polysciences Inc., Eppelheim, Germany). Cells were treated with 0 or 100 ng/ml IL-6 for 24 h. Cells were lysed by addition of 20 µl of Passive Lysis Buffer (Promega) per well. Activity was determined with the Luciferase Reporter System (Promega) in combination with a Mithras LB 940 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA extraction was performed usingpeqGOLD TriFast Reagent (PEQLAB, Erlangen, Germany). RNA was further purified using chloroform/isopropanol extraction. Reverse-transcription of RNA was achieved using the iScript™ cDNA Synthesis Kit (BIO-RAD, Munich, Germany). qRT-PCR analyses were accomplished using the iCycler-System (BIO-RAD) and the Absolute qPCR SYBR Green Fluorescein Mix (Thermo Scientific, Schwerte, Germany). Data were normalized to the housekeeper hypoxanthine-guanine phosphoribosyl-transferase (HPRT). HPRT was chosen as housekeeper gene as its expression appeared independent from an IL-6 stimulus. The delta-Ct method was used for quantification of relative transcript concentrations.15 Primer sequences for human SePP, CP, DIO1, SELS, GPX-1,-2,-4, and HPRT are provided in the supplemental, along with the experimental data (Ct values) of the qRT-PCR analyses.

Western Blot analyses

After incubation periods of 48 h, cells were harvested in lysis buffer (50 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerin). Protein concentrations were quantified by Pierce BCA Protein Assay Kit (Thermo Scientific) using BSA as standard. Samples of equal protein concentration were diluted with 4x sample buffer (200 mM Tris-HCl, pH 7.5; 50% glycerin; 4% SDS; 0.04% bromophenol blue and 125 mM DTT). Samples were size fractionated by SDS-PAGE and blotted by semi-dry transfer onto nitrocellulose membranes (Optitran, Schleicher & Schuell, Dassel, Germany). Antibodies against SePP (1:2,500 dilution, ICI – immunochemical intelligence GmbH, Berlin, Germany), ceruloplasmin (CP) (1:2,000 dilution, Abcam, Cambridge, UK), GPX-1 (1:1,000 dilution, Abcam), GPX2 (kind gift of Dr. A. Kipp, DIfE, Potsdam, Germany), GPX4 (1:1,000 dilution, Abcam), DIO1 (1:1,000 dilution, kind gift of Dr. G. Kuiper, Rotterdam), SELS (1:1,000 dilution, Sigma Aldrich) and beta-actin (1:25,000 dilution, Roche, Germany) were used and quantified by ECL™ Western Blotting Detection Reagents (GE Healthcare, UK).

Enzyme activity assays and SePP ELISA

SePP concentrations were quantified from conditioned cell medium by a SePP-specific ELISA (Selenotest®, ICI). From cell homogenate, the total enzymatic activity of GPX was measured using hydrogen peroxide (H₂O₂) as substrate.16 DIO activity was assayed by a non-radioactive test as described recently.17

Statistics

Statistical analyses were performed using GraphPad Prism v.4.0 (GraphPad Software Inc., San Diego, USA). The results are represented as means ± SEM (standard error of the mean). The number of replicates is given in the figure legends. For normal distributed data, two group comparisons were conducted using Student’s t-test, otherwise Mann-Whitney-test was applied. In case of non-normally distributed data (> 2
groups), the Kruskal-Wallis-test was used. Statistical significance was assigned if $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***)

**Results**

**IL-6 regulates SePP and CP expression in opposite directions**

The Cu transport protein CP is a well-characterized positive APP while the Se transport protein SePP is described as a negative APP. Both CP and SePP are produced by hepatocytes. As IL-6 represents a central pro-inflammatory cytokine in sepsis, its direct effects on these two trace element transporters were compared. Secretion of SePP in HepG2 cells becomes strongly increased by addition of 100 nM sodium selenite ($Na_2SeO_3$) to the culture medium (Fig. 1A). Co-application of IL-6 diminishes the Se-dependent increase of SePP concentrations in the culture supernatant strongly but not completely (Fig. 1A). The suppressing effect of IL-6 on selenite-induced SePP biosynthesis proceeds in a dose-dependent manner (Fig. 1B), and in both hepatoma cells tested (Fig. 1C). In comparison, the concentration of CP remains unaffected by Se (Fig. 1A), but increases in response to IL-6 (Fig. 1B), as known from literature.

QRT-PCR analyses indicate qualitatively similar effects on the transcript levels. Steady-state concentrations of CP mRNA increase in response to IL-6 treatment strongly, whereas SePP mRNA levels are diminished in response to IL-6 (Fig. 1D).

**Effects of IL-6 on intracellular selenoproteins DIO1 and SELS**

Besides SePP biosynthesis, selenite supplementation of the culture medium is likely to increase also other selenoproteins. Relatively low expression of DIO1, the major DIO isozyme expressed in hepatocytes, is detected by Western blot analysis under Se-deficient conditions; as expected, its expression strongly increases in response to Se (Fig. 2A).

**Fig. 1. Regulation of Ceruloplasmin (CP) and Selenoprotein P (SePP) expression by Interleukin-6 (IL-6).** Hepatic cells were incubated in presence (+) or absence (-) of 100 nM sodium selenite ($Na_2SeO_3$) for 24 h. (A) Western Blot analysis ($n = 6$) indicates Se-independent induction of CP expression by IL-6 and suppressing effects of IL-6 on Se-dependent SePP biosynthesis in HepG2 cells. (B) The suppressive effect of IL-6 on secreted SePP levels are dose-dependent ($n = 2$) (C), and similarly seen in both HepG2 and Hep3B hepatocytes. (D) QRT-PCR analyses indicate that the increase in CP and decrease in SePP secretion are accompanied by respective changes on the mRNA level ($n = 6-9$).

**Fig. 2. Regulation of deiodinase type I (DIO1) and selenoprotein S (SELS) expression by interleukin-6 (IL-6).** HepG2 cells were incubated and analysed as described in Fig. 1. Beta-actin (ACTB) was chosen as intracellular control protein. (A) Western Blot analysis shows Se-dependent induction of immunoreactive DIO1 protein levels; the induction is diminished by co-incubation with IL-6. (B) The suppressive effect of IL-6 on DIO1 expression is similarly reflected in the enzyme activity, and (C) on the mRNA level ($n = 9$). (D) In comparison, mRNA expression of SELS was unaffected by IL-6. The same applies to (E) the immunoreactive SELS concentrations, which were strongly responsive to Se supplementation, but not to IL-6.

In the presence of IL-6, the Se-dependent induction of DIO1 expression is diminished (Fig. 2A), in line with a reduced DIO1 enzymatic activity (Fig. 2B). This effect is mirrored on the
transcript level, and DIO1 steady-state mRNA concentrations are reduced in the presence of IL-6 as compared to control (Fig. 2C). In comparison, SELS transcript concentrations remain unaffected by IL-6 treatment under these conditions (Fig. 2D). This notion is supported by relatively constant SELS protein levels in a Western blot analysis, where selenite supplementation induces SELS protein levels profoundly while no modulatory effect of IL-6 on SELS expression in the presence or absence of supplemental Se is detected (Fig. 2E).

**Isozyme-specific regulation of hepatic GPX expression by IL-6**

Total GPX activity in HepG2 cells using H$_2$O$_2$ as substrate increased in response to selenite supplementation and IL-6 stimulation (Fig. 3A). Secreted GPX3 activity was not detectable. In order to obtain GPX isozone-specific information, Western blot and qRT-PCR analyses were performed. Expression of immunoreactive GPX1, GPX2 and GPX4 protein levels were largely unaffected by IL-6 stimulation under the culture and Western Blot conditions (Fig. 3B). On the transcript level, GPX1 mRNA levels was unaffected by IL-6 treatment.

**Fig. 3. Regulation of glutathione peroxidase (GPX) expression by interleukin-6 (IL-6).** HepG2 cells were treated as described in Fig. 1. (A) Total GPX activity increases strongly in response to selenite, and the induction is further increased upon IL-6 treatment. (B) Western Blot analysis indicates strong effects of selenite on immunoreactive GPX protein levels, but no consistent effects of IL-6. (C) QRT-PCR analyses ($n$ = 8-9 per transcript) however indicate isozone-specific effects of IL-6 on GPX mRNA concentrations; while GPX1 mRNA remains unaffected, GPX2 mRNA levels increase and GPX4 mRNA levels decrease.

However, transcript concentrations of GPX2 were up-regulated whereas transcript levels of GPX4 were down-regulated in response to IL-6 (Fig. 3C). These findings indicate a particular pattern of gene-specific effects of IL-6 on the expression of hepatic GPX isozymes.

**Reporter gene analyses indicate direct effects of IL-6 on selenoprotein promoter activities**

Intracellular IL-6 signaling is mainly mediated via the transcription factor STAT3 that binds to respective STAT3 responsive elements within control regions of IL-6 target genes. In order to analyze direct effects on hepatic selenoprotein gene expression, luciferase reporter gene constructs were generated comprising approximately 1 kb of DNA directly upstream of the transcription start sites of the different selenoprotein genes. A synthetic STAT3 response element-containing plasmid was used as positive control (Fig. 4A), and the empty vector as negative control (Fig. 4B). The two human hepatoma cell lines HepG2 and Hep3B were chosen as test models as they are known to have maintained their responsiveness to IL-6 in culture.
As expected, IL-6 dose-dependently induces reporter gene expression from the STAT3-positive control vectors in both HepG2 and Hep3B cells (Fig. 4A), while not affecting the empty vector (Fig. 4B). Expression from a reporter gene containing the core SePP promoter sequence declines in response to IL-6 treatment in both hepatoma cell lines (Fig. 4C). A similar suppressive effect is observed when the reporter gene construct containing the core promoter sequence of DIO1 is tested (Fig. 4D). In comparison, the reporter gene containing the GPX2 core promoter becomes specifically induced by IL-6 treatment in both cell lines tested (Fig. 4E), while the GPX4 promoter construct is repressed (Fig. 4F), and the GPX1 promoter remains unaffected (not shown). These findings are in line with the effects observed on the mRNA and protein levels and indicate direct and specific transcriptional effects of IL-6 on the hepatic selenoproteome.

**Discussion**

Our data indicate gene-specific effects of IL-6 on the hepatic expression of a number of selenoprotein-encoding genes. An unexpected complex picture of effects emerges in response to a single prominent cytokine (Fig. 5). As the liver has been identified as the central organ controlling systemic Se metabolism and SePP-mediated Se transport, our results are of specific relevance for the therapeutic attempts of Se substitution and supplementation in the clinics. Especially in critically ill patients, the results from other studies are controversial with respect to benefits of Se supplementation. Sepsis is a severe, life-threatening disease characterized by a strong immune response to overcome the infection and challenging for the whole organism. The inflammatory reaction involves an excessive production of reactive oxygen species (ROS) and pro-inflammatory cytokines to control the migration and activity of immune cells, and to adapt central metabolic pathways in the major organs to the health threat. Under inflammatory conditions, the iron (Fe) redistribution away from the circulation into intracellular storage sites leading to a condition known as “anemia of chronic disease” represents a well-characterized example of such an adaptive program. The direct regulation of the hepatic hormone hepcidin by IL-6 proved of central importance for this targeted redistribution of Fe. Important physiological reasons for this metabolic adaptation are the attempt to reduce availability of the essential trace element to the invading pathogens while supporting endogenous hemoglobin synthesis at the same time. Therapeutic iron supplementation in severe disease thus constitutes a controversial strategy as it is potentially supporting both the host and the invaders. A similar redistribution of zinc (Zn) as another immune-relevant essential trace element is described in critical illness raising another challenge for nutritional support of severely diseased patients.

In contrast, the Cu transport protein CP is known as a positive APP in blood that increases under inflammatory conditions and in sepsis. Three distinct IL-6 response elements have been identified in the upstream region of the CP gene. The IL-6-stimulated CP expression involves FOXO1, a transcription factor known to contribute to the anti-oxidative response. The reason for reducing intracellular levels of Cu as an acute phase response by increased CP secretion from hepatocytes and reticulo-endothelial cells is not fully understood. CP may constitute a positively regulated APP as it has the potential to scavenge oxygen-derived free radicals in the circulation for protecting vessels and endothelial cells. Moreover, high Cu concentrations are known for their bactericidal properties. In addition, CP is capable of oxidizing toxic ferrous Fe(II) to more stable ferric Fe(III) ions thereby further reducing ROS concentrations and ROS-mediated damage. Similarly, a protective function for the Se-transport protein SePP has been described under inflammatory conditions because of its anti-oxidative and peroxynitrite-scavenging properties in human plasma. In parallel to CP, also SePP is positively regulated by the transcription factor FOXO1. Yet, in inflammation, previous studies have indicated that SePP qualifies as a negative APP and a sensitive marker for septic shock. Our data verify the inverse regulation of hepatic CP and SePP expression, and support the classification of SePP as a negative APP by demonstrating that its transcription is a direct target of IL-6 signaling in human hepatocytes. Obviously, IL-6 affects blood Cu and Se concentrations in mirror image direction by direct genomic effects in hepatocytes causing decreased SePP and increased CP biosynthesis in parallel. This may cause Se deficiency and low expression of selenoproteins in SePP-dependent organs.

![Fig. 5. Schematic summary of the IL-6 dependent changes in the hepatic selenoproteome. Under normal conditions, the available](Image)
The question arises on why these two trace element carriers are regulated in opposite direction?

Several hypotheses can be put forward in order to answer this question. While Cu is an essential micronutrient for most organisms, selenoproteins are found in only one-fourth of the bacteria genetically analyzed to date indicating that most microbes grow Se-independently. In contrast to CP, the decline in SePP expression and the reduced secretion in response to IL-6 is therefore rather driven by a selective advantage for the host organism and not as a defense strategy targeting the invaders, as is currently assumed for the aforementioned Fe-dependent anemia in inflammation.

It can be safely assumed that intracellular Se concentrations increase when hepatocytes down-regulate SePP biosynthesis and secretion in response to IL-6. This has been observed in Sepp knockout mice where Se concentrations increased specifically in liver whereas they declined in other organs including serum highlighting a relatively profound Se loss of hepatocytes by SePP secretion under normal conditions. The selective down-regulation of hepatic SePP by IL-6 improves available energy and positively affects Se availability in the cell, thereby facilitating production of other intracellular selenoproteins. Hepatic GPX2 may constitute such an urgently needed central hepatic selenoenzyme positively benefitting from reduced SePP biosynthesis. The up-regulation of GPX2 in response to IL-6 may be meaningful to reduce intracellular oxidative stress in hepatocytes, in a similar way as recently demonstrated for its role in gastrointestinal cells. It remains to be determined whether the increased Se concentrations are also converted to more excretion products destined for Se disposal.

Quantitative analyses of the urinary selenocompounds from severely diseased patients may be suitable to identify such a potential contribution of IL-6 to the pattern of selenosugars secreted by humans apart from the influence of age and obesity.

Previous in vitro analyses already indicated suppressing effects of interleukin-1β and tumor-necrosis-factor α on SePP expression. These two cytokines are secreted mainly in the early stages of infection, while IL-6 becomes the main regulator of the inflammatory response with time. It thus appears as if the down regulation of hepatic SePP production and secretion is both an early and long lasting event during severe inflammatory conditions, potentially intended to be operative during all stages of a severe inflammatory disease.

Our findings on reduced expression of the selenoenzyme DIO1 in liver cells upon IL-6 stimulation is in line with similar observations made before in cell culture, rodent models of sepsis and human patient tissues. Notably, our studies focused on IL-6-mediated effects on Se-replete cells, as also in the patients, hepatocytes are unlikely to experience immediate Se deficiency in inflammatory diseases for the reasons given above. This may explain some discordant findings in comparison to studies analyzing serum-free Se-deprived hepatocytes in culture.

SELS expression in hepatocytes was only marginally affected by IL-6. This is in contrast to the findings of a selective up-regulation of SelS expression in response to LPS in murine models of acute septic shock. A reason for this difference may lie in the different pathways activated by IL-6 in comparison to LPS, and in the difference in the cell types analyzed as regulation of SELS in hepatocytes may differ from SELS gene regulation in cells with immunological origin. Consequently, quantitative immune-based histochemical analyses with tissues from model systems or diseased patients are needed in order to better understand cell-type specific differences in the acute phase regulation of selenoproteins in critical disease.

Conclusions

Collectively, we observe a strong negative impact of IL-6 on hepatic SePP and DIO1 expression. Both selenoproteins have been implicated in important and mortality-relevant metabolic adaptations in patients, i.e., the low T3 syndrome and the systemic Se deficiency in critically-ill patients. It remains to be tested whether these two metabolic adaptations are affected in studies pharmacologically impairing IL-6 signaling in human patients by targeted therapies (e.g. by tocilizumab treatment), e.g. in rheumatic diseases. To the best of our knowledge, such therapeutic interventions have not yet been combined with the analyses of Se and Cu biomarkers.

In case our hypothesis on the direct effects of IL-6 on selenoprotein expression holds true in patients, it opens a promising adjuvant therapeutic options for severe diseases in which the deregulation of selenoprotein expression and thyroid hormone concentrations contribute to disease severity and mortality risk. This question has just received additional relevance in view of the dependence of STAT3-mediated signaling by H2O2-dependent modification of peroxiredoxin-2 thereby potentially closing a feedback loop with Se status and GPX expression in the acute phase response. While a fast down regulation of SePP biosynthesis and secretion from hepatocytes may have evolutionarily been advantageous, the prolonged down regulation of circulatory SePP and Se availability to other tissues, which proceeds thanks to our modern medical opportunities on the intensive care units, may indeed impose health risks if not counteracted by active and targeted Se supplementation to overcome Se deficiency and normalize Se transport and thyroid hormone metabolism.

Acknowledgements

We thank Carola Geiler and Kristin Fischer for excellent technical assistance. The research was supported by the German Research Foundation (GraKo 1208; Scho 849/4-1, Re
Notes and references