Regulation of urea synthesis during the acute phase response in rats

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PAPERS ON WHICH THIS THESIS IS BASED


INTRODUCTION

Catabolism is a serious clinical problem in patients with various states of active inflammation. The catabolism and loss of tissue nitrogen (N) is a result of proteolysis, and this loss of tissue protein presents a threat to the integrity of the organism. The negative N balance may be caused by diminished diet protein intake, activated stress-hormones with increased protein break-down, and amino acid release into the blood-stream, and/or an up-regulated hepatic removal of amino-N via urea synthesis. This latter hepatic component of inflammatory catabolism is considered to play a primary role by depleting the blood-free amino acid pool and favouring tissue protein break-down over build-up. Therefore, it is patho-physiologically and potentially therapeutically important to identify the mechanism of this regulation of urea synthesis that occurs during inflammatory loss of body N. This has been the main focus of this PhD thesis.

It has earlier been shown that a systemic inflammatory response, the so-called acute phase response, following administration of lipo-polysaccharides (LPS) is associated with an up-regulation of urea synthesis in rats. A likely mediator of this effect is the cytokine tumor necrosis factor alpha (TNF-α); TNF-α increases during inflammation and is a central direct mediator of the acute phase response in rats.

The aim of this thesis has been to characterise the regulation of hepatic N elimination via urea from urea cycle enzyme gene expression to regulation of whole-body urea synthesis during different phases of the TNF-α-induced acute phase response. This work is based on and extends previous studies on the effects of LPS by describing the time-course, the effect of supposed urea synthesis regulators like different hormones and cytokines, and by determinations of the protein levels of the urea cycle enzymes.

BACKGROUND

Urea synthesis

Urea synthesis is an essential function (i.e. essential for maintenance of health and sustenance of life) that takes place only in the liver. The liver plays a central role in amino acid metabolism during anabolism and catabolism. When amino-N is available in excess relative to its use for tissue protein build-up, it is eliminated from the body via the synthesis of urea; the whole-body physiological substrate for urea synthesis is α-amino-N[1, 2]. There are only small variations in the amount of N lost in the faeces, from the skin, and via urinary NH₃ excretion, and the regulation of urea production is therefore the key to N balance [1]. As urea synthesis is an irreversible process and as urea cannot be broken down by mammalian cells and reutilised, although a small amount of urea is hydrolysed in the gut by bacterial ureases and recycled back to urea as NH₃ [3], the production of urea is the final step in the body’s elimination of N. Thereafter, urea is excreted in the urine.

The urea cycle

The cycle of enzymatic processes leading to urea formation from NH₃ and CO₂ was discovered by Krebs and Henseleit in 1932. The cycle consists of five steps catalysed by the two initial mito-
Chondrial enzymes carbamoyl phosphate synthetase (CPS) and ornithine transcarbamylase (OTC), followed by the three cytoplasmic enzymes argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and arginase (ARG) [4]. The steps of the urea cycle at which the urea cycle rate may be controlled and limited are at the first and third enzymes, CPS and ASS. CPS is the flux-generating urea cycle feeder enzyme determining the flux into the cycle, and ASS is ultimately the rate-limiting cycle enzyme, because this enzyme has the lowest in vitro $V_{\text{max}}$ among the five enzymes. Under physiological conditions, the cycle flux is controlled by the activity of CPS. However, in a near-substrate-saturated situation, the capacity of the cycle is increasingly determined by the $V_{\text{max}}$ of the ASS. At any moment, the activity of the CPS is determined by the concentration of its obligatory allosteric activator N-acetyl-glutamate. The activity of the CPS is also subject to long-term regulation by induction of the transcription of the CPS gene. The ASS is subject to regulation only by induction of its gene [5].

**Regulation of urea synthesis**

From a whole-body point of view, the rate of the urea synthesis is mainly determined by the blood concentration of alpha-amino N, which is the physiological substrate for urea synthesis; the rate of urea synthesis is highly substrate dependent. However, also a non-substrate regulation of urea synthesis takes place [6]. Functional liver mass is crucial for an intact capacity of urea synthesis, and in fact, urea synthesis is a reliable quantitative measure of functional liver mass both in the rat and in man [2, 7]. Also, glucose and the intake and composition of food are important regulators of urea synthesis [8-10]. Other regulators are a large number of hormones. The most important hormone in this context is glucagon that exerts both moment-to-moment regulation via its effects on N-acetyl-glutamate and long-term regulation via its induction of the urea cycle genes [11-13]. Cortisol and adrenaline also up-regulate urea synthesis [14-16]. Growth hormone and insulin-like growth factor-1 (IGF-1) down-regulate urea synthesis [17, 18]. The general picture thus seems to be that the co-called stress hormones act to increase urea synthesis and the so-called anabolic hormones have the opposite effect.

To study regulation of urea synthesis in vivo, it is necessary to standardise the process rate for the strong effects of substrate blood amino acid concentration. To this end, the process rate in rats can be substrate-saturated by means of alanine infusion. This allows measurement of the maximum rate, i.e. the capacity of urea-N synthesis (CUNS). Any change in this capacity reflects non-substrate regulation of urea [19]. The importance of events of this function on a gene level can be assessed by quantifying the hepatic urea cycle enzyme mRNA levels. Most previous studies have shown experimental or physiological changes in the expression of urea cycle enzyme genes to parallel the changes in CUNS [17, 20] (or visa-versa). This may, however, not be the case during inflammatory regulation of urea synthesis [21]. To approach a better understanding of the formation and function of urea cycle enzymes, it is, therefore, in such situations, necessary also to study other determinants of the cycle activity, e.g. urea cycle enzyme proteins in liver tissue or the catalytic activity of such proteins.

**Acute phase response**

The acute phase response, a component of the innate immune system, is the systemic response to tissue injury, inflammation, infection, and cancer [22]. The acute phase response results in a large increase in the hepatic synthesis and release of so-called positive acute phase proteins [23, 24]. These proteins serve various functions during initiation, maintenance, and overcoming of the acute phase response in an attempt to restore homeostasis [25]. The synthesis and export of the positive acute phase proteins take over most of the liver’s synthetic capacity [23]. The amino acids required for this protein synthesis derive in part from reduced synthesis of proteins that are less important for host defence (so-called negative acute phase proteins) and in part from muscle protein degradation [25, 26].

The acute phase proteins determined in this study are among the most specific indicators of the acute phase response in the rat [27]. Alpha-2-macroglobulin (α2MG) is known to display the strongest response during an acute phase response since the increase in protein concentration reflects de novo synthesis as almost no α2MG protein is present under non-induced conditions [28]. Newly synthesised alpha-1-acid glycoprotein (α1AGP) and haptoglobin are secreted into an already existing pool of α1AGP and haptoglobin in serum. Albumin is one of the so-called negative acute phase proteins that decreases during the acute phase response [23].

An acute phase response is a complex immune reaction involving cascades of cytokines, chemokines, and signalling pathways [29]. The pro-inflammatory cytokines TNF-α, interleukin-1β (IL-1β), and IL-6 are key factors in initiating and organising the acute phase response. These cytokines are components of a large signalling network, and they are able to regulate the production of and modulate the action of other cytokines [25]. The cytokines are produced by a variety of cell types, the most important sources being macrophages and monocytes at inflammatory sites [24].

**Tumor necrosis factor alpha**

TNF-α is a cytokine produced mainly by macrophages but also by various lymphoid cells [30]. It exerts its biological functions via interactions with two different receptors, TNF-R1 expressed in most tissues and TNF-R2 typically found in immune cells [30, 31]. TNF-R1 is activated by soluble TNF-α, whereas TNF-R2 activation requires the binding of membrane-bound TNF-α [32]. After binding to the receptors, activation of intracellular signalling pathways is initiated. TNF-α is able to activate several intracellular pathways to regulate inflammation, cell death, and proliferation, e.g. the IKK/NF-κB pathway being particularly important during inflammatory responses by protecting hepatocytes from TNF-induced cell death and regulating the transcription of pro-inflammatory mediators in Kupffer cells [31, 33]. NF-κB is a so-called transcription factor that binds to specific DNA sequences, thereby controlling the transcription of genetic information from DNA to mRNA. NF-κB is held in an inactive state in the cytoplasm by IκB (inhibitor of kappa B) proteins. After activation of the TNF-α receptor, IκB is phosphorylated by an IκB kinase (IKK) complex and subsequently degraded, after which NF-κB is liberated and is now able to enter the nucleus to initiate transcription [30].

TNF-α displays a wide range of biological activities, and most cells show TNF responsiveness. TNF-α rises during inflammation [34, 35], and large amounts of TNF-α are released in response to LPS [36, 37]. TNF-α in itself is a central direct mediator of the acute phase response; TNF injection in rats induces acute phase proteins [38, 39]. TNF-α is considered to be a key player in the development of septic shock. Administration of TNF-α produces a variety of metabolic and hormonal effects, similar to changes...
associated with septicaemia and acute tissue injury, suggesting that TNF-α is a communicator between the functions of the immune system and the functions of the endocrine system [40-42]. TNF-α induces, among other things, protein breakdown and catabolism [43-46] and stimulates liver amino acid uptake in the rat [42]. Moreover, prolonged exposure to low TNF-α concentrations results in anorexia [47] and cachexia [48]. Injection of high doses of TNF-α produces a septic-like response with systemic and visceral haemodynamic changes and hepatocellular dysfunction measured as a decrease in galactose clearance [49, 50]. Low doses, such as those used in our studies do not, however, induce these haemodynamic changes, but still, hepatocellular function is affected [51].

**Interleukin-6**

IL-6 is produced by various types of lymphoid and non-lymphoid cells [52]. Like TNF-α, IL-6 is a multifunctional cytokine involved in the regulation of haemopoiesis and immune responses [52, 53]. However, when binding to its IL-6 receptor, it activates a different transcription factor called NF-IL-6, which translocates into the nucleus after phosphorylation and mediates the transcription of specific genes. A de novo synthesised nuclear factor NF-IL-6β contributes to this gene transcription [25]. During inflammation both IL-6 and TNF-α rise and both are central players in the initiation and organisation of the acute phase response [34, 54, 55]. Yet, IL-6 is found to be the chief stimulator of the production of most acute phase proteins [24, 28, 53]. IL-6, however, not only induces an acute phase reaction, but at the same time it also inhibits the propagation of pro-inflammatory cytokine signalling through a negative feedback loop, which serves to control and resolve inflammation [25]. In this way IL-6 down-regulates the expression of TNF-α, which otherwise acts as a mediator in the propagation of the inflammatory response and in itself increases IL-6. In addition to these complex immunological effects, IL-6 has various metabolic effects. IL-6 acutely activates the hypothalamic-pituitary-adrenal axis by stimulating the corticotropin-releasing hormone (CRH) neuron, which leads to increased secretion of adrenocorticotropic hormone (ACTH) and cortisol [56]. Likewise, IL-6 affects carbohydrate metabolism by transiently increasing glucagon followed by increased glucose and eventually insulin levels [57]. In the light of these mechanisms, it is not surprising that IL-6 also induces muscle proteolysis in vivo [58].

**Urea synthesis and the acute phase response**

During an acute phase response, hepatic protein synthesis is markedly increased [59, 60] and profoundly reorganised [23]. This involves gene events on the transcription level and stimulates the synthesis of proteins involved in defence functions (the so-called acute phase proteins), while the expression of genes for other groups of hepatic proteins are shut down (the so-called negative acute phase proteins, classically albumin).

In this situation, it seems to be advantageous for the whole-body N economy that the liver produces less urea relative to the prevailing amino-N concentration. This might involve down-regulation of the urea cycle enzymes’ gene expression, mirroring the up-regulation of the acute phase proteins’ mRNA levels and, more importantly, a resulting decrease in the in vivo capacity of urea synthesis (CUNS). Accordingly, the urea cycle enzyme genes have previously been found to be “negative acute phase protein genes”, as demonstrated after LPS administration in rats [21, 61]. However, previous studies have also and paradoxically shown that both clinical (i.e. during human disease states) and experimentally induced, active inflammation up-regulates urea synthesis, which promotes N removal from the body [21, 62]. Consequently, the acute phase response presents a critical catabolic event that, together with the accelerated proteolysis, results from increased waste of amino-N via urea synthesis despite the increased need for amino-N for incorporation into the acute phase proteins.

The liver’s role in inflammatory catabolism is comparable with its role after trauma and surgery, a phenomenon previously termed “the hepatic catabolic stress response” [63] and ascribed mostly to the effects of the “stress hormones”. However, it remains unknown which mechanisms are operative during this phenomenon in inflammation. Glucagon and cortisol could also be mediators during inflammation [12, 14]. A link between the sympathetic-adrenal system and the immune system has been suggested; many cytokines increase plasma concentrations of cortisol and glucagon (e.g. during surgical stress). Accordingly, cytokines of the acute phase cascade could be responsible for the hepatic catabolic stress response just as they are for most of the changes of hepatic protein metabolism during inflammation [64]. So far, however, only an up-regulatory effect of IL-1β on urea synthesis has been described [65], and the effect was shown to be glucocorticoid-dependent, i.e. only an indirect effect. The central pro-inflammatory cytokines TNF-α and IL-6 are capable of inducing the amino acid flux shift from the muscle to the liver observed in vivo during an acute phase response [64]; they increase proteolysis in the muscles and have been shown to increase the amino acid uptake by the hepatocytes. TNF-α and/or IL-6 might, therefore, up-regulate urea synthesis and exert their catabolic effects partly via this mechanism. However, no study on the regulatory effects of TNF-α and IL-6 on urea synthesis has so far been reported.

**HYPOTHESES AND AIMS**

The aims of this study were to study the role of TNF-α and IL-6 as mediators of changes in the regulation of urea synthesis in the experimental TNF-α- or IL-6-induced acute phase response; to identify the mechanism responsible for changes in the regulation of urea synthesis during the acute-phase response; and to study the time-course of changes in the regulation of urea synthesis after the experimental acute phase response.

We used four methods to study the regulation of urea synthesis at different levels:

- Urea cycle enzyme mRNA levels in liver tissue (gene regulation)
- Urea cycle enzyme proteins in liver tissue (translational regulation)
- The capacity of urea nitrogen synthesis (CUNS) (in vivo regulation)
- Known hormonal regulators of CUNS

We hypothesised that

- TNF-α induces up-regulation of CUNS
- IL-6 induces up-regulation of CUNS
- Changes in the in vivo urea synthesis parallel changes in the mRNA and protein levels of the urea cycle enzymes

Insight into the interplay between hepatic production of acute phase proteins and ureagenesis and identification of possible
Mediators are important for the mechanistic understanding of catabolism during inflammation and for the elucidation of the possibilities of intervening in the process.

**METHODOLOGICAL ASPECTS**

**Study design**
The study consisted of four sub-studies conducted 1, 3, 24, and 72 h after injection of either placebo (saline) or TNF-α and 1 sub-study conducted 3 h after injection of either placebo (saline) or IL-6. The experimental protocol was approved by the Danish Research Animal Committee, Copenhagen, Denmark, according to license number 2006/561-1099. Female Wistar rats (body weight 200–210 g) were housed at 21 °C ± 2 °C with a 12-h artificial light cycle. Two to three animals were housed in each cage, with free access to tap water and controlled access to standard food. Following anaesthesia via the inhalation of 2–3% isoflurane, 0.2 ml of saline 0.9%, 25 µg x kg-1 of recombinant rat TNF-α (rTNF-α) or 125 µg x kg-1 recombinant human IL-6 (rhIL-6) that had been dissolved in 0.2 ml of isotonic saline were intravenously injected into one of the tail veins of each rat. This is a highly reliable method for administering substances in rats and ensures 100% bioavailability. In the studies conducted 24 and 72 h after injection of TNF-α, cage-to-cage pair feeding of animals was instituted, the control animals being given the same amount of food as that consumed by the intervention animals. Proper pair feeding would have been desirable, but because it is not advisable to house rats separately, we chose to settle for cage-to-cage feeding. Each rat was weighed before substances were administered and again before the experimental procedures. In approximately half of the animals, blood was collected for analyses and the liver was excised. In the other half of the animals, CUNS was determined as previously described [19].

**Choice of methods for analysing liver tissue and blood**
The methods used for the analyses of liver tissue were chosen on the basis of the fact that liver tissue and hepatocytes contain huge amounts of proteins. Accordingly, there is a potential risk of cross-reactivity between similar proteins when analysing the samples for specific proteins. Therefore, it is important to make sure, as far as possible, that only the protein of interest is determined. To this end, Western blot is a reliable method used to detect specific proteins in a sample of homogenised tissue. Before detecting the proteins by applying antibodies specific to the target protein, the proteins of the sample are separated by gel electrophoresis to actually see whether the detected protein has the expected molecular size. In the blood, proteins like insulin, cytokines, etc. are present in small concentrations relative to the other components of the blood. In the present study, we used commercial kits developed to detect these substances in the blood with high sensitivity and specificity and validated for use in the rat.

**Liver tissue**

**mRNA determination**
We chose to measure mRNA levels of the five urea cycle enzymes, the acute phase proteins, and IGF-1 and insulin-like growth factor binding proteins (IGFBPs) in liver tissue by slot blot hybridisation as previously described in detail [66]. When using slot blot hybridisation, it is not possible to actually verify whether the mRNA sequence of interest is the only mRNA sequence determined, as the total amount of mRNA is administered in a well and not separated by gel electrophoresis prior to applying the probe. If we incorrectly detect two molecules of different sizes, they will appear as a single dot and not as two distinct bands after protein separation in a gel. However, all the cDNA probes used were previously validated using Northern Blots, and we therefore felt secure using the method of dot blot hybridisation. The mRNA results are given in relative levels compared to control animals to avoid potential intervention-induced changes in the housekeeping genes usually used.

**Western Blots**
We used the well-established Western blot method to detect protein levels of the urea cycle enzymes CPS and OTC in liver tissue. The obtained CPS1 and OTC antibodies showed high affinity to their antigens, reacting with rat CPS1 and OTC at low concentrations. The blots revealed the two proteins as single distinct bands in the membrane at the predicted molecular weight.

**Blood analyses**

**Urea and α-amino-N**
Blood urea was measured via the urease-Berthelot method [67], whereas total blood α-amino-N was measured via the dinitrofluorobenzene method [68]. CUNS was calculated as the body accumulation of urea corrected for intestinal hydrolysis [19]:

\[ \text{CUNS} = (dC_u/\text{dt}) \times 0.63 \times BW \times 1/(1 - 0.14) \]

Where (dC_u/\text{dt}) is the slope of the linear regression analysis of serum urea on time during steady state, 0.63 x BW (body weight) is the distribution volume of urea, and 1/(1 – 0.14) is the correction factor for intestinal hydrolysis and recycling of resulting ammonium into urea.

**Acute phase proteins**
The prevailing rat acute phase proteins α2MG, haptoglobin, α1AGP, and albumin concentrations in serum were evaluated using specific rat enzyme-linked immunosorbent assays (ELISA). All the assays have been validated for use in rats.

**Corticosterone**
Corticosterone is the active glucocorticoid in the rat. Plasma corticosterone concentrations were assessed using a specific rat radioimmunoassay (RIA) in the studies conducted 3 and 24 h after TNF-α injection. In the other three studies, plasma corticosterone concentrations were assessed using an enzyme immunoassay (EIA) specific for rat corticosterone. At the beginning of the project, we made use of another laboratory for corticosterone analyses, but later we implemented the analysis in our own lab. As we did not have facilities for handling of radioactive materials at our disposal, we had to use another, isotope free method. Our enzyme-immunoassay matched the RIA with regard to both sensitivity and specificity.

**Glucagon**
Plasma glucagon was measured by wick chromatography in the studies conducted 3 and 24 h after TNF-α injection [69]. In the studies conducted 1 and 72 h after TNF-α injection and 3 h after IL-6 injection, plasma glucagon was measured using another method of RIA [70]. In the present study we used plasma glucagon and corticosterone levels to identify a potential hormonal effect on urea synthesis within each sub-study. Accordingly, our purpose was not to compare the levels between the different studies, and therefore, using two different methods should be of no importance.

**Insulin, glucose and the HOMA index**

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Serum insulin was measured using an ultrasensitive rat insulin ELISA, and plasma glucose was determined by a routine analytical method. The HOMA index was calculated in accordance with a previously described model [71].

Cytokines
Plasma IL-6 was determined using a specific rat IL-6 immunoassay. In the studies conducted 3 and 24 h after TNF-α injection and in the 3 h IL-6 study, plasma TNF-α was determined using xMAP® technology. In the 1 and 72 h studies after TNF-α injection, plasma TNF-α was determined using a specific rat TNF-α immunoassay. The advantage of the bead-based multiplexing xMAP® technology over the immunoassay is that it delivers more data in less time because of its ability to quantify a large number of cytokines in a small sample simultaneously. This is very important in rat studies in which sample volumes are limited. Accordingly, the xMAP® technology implies a reduction in time and costs. However, using the xMAP® technology sensitivity may be compromised because of the high number of beads per well. Also, there is a possible cross-reactivity between antibodies. Therefore, and as we were only interested in a limited number of cytokines, we chose to use a specific rat TNF-α immunoassay for the latest samples.

IGF-system
Total serum IGF-1 was measured after acid-ethanol extraction by an IGF-1 sandwich immunoassay. IGFBPs were measured by SDS-PAGE and Western ligand blot (WLB) analysis as described elsewhere [72, 73].

In vivo rate of urea synthesis
The rate of urea synthesis in rats can be determined as the accumulation of urea in nephrectomised rats corrected for accumulation of urea in total body water and for hydrolysis of urea in the gut [19]. This method has been validated and applied to studies of the role of urea synthesis for N homeostasis in a variety of experimental diseases [20, 74-76] and investigations of hormones and peptides with effect on urea synthesis [13, 14, 16, 17, 65]. Urea is excreted in the urine, and because quantitative collection of urine in rats is difficult, nephrectomy is performed before determination of urea synthesis rate. This does not influence the rate of urea synthesis within the time limits of the investigation [19]. The space of distribution of urea is equal to total body water [77]. In rats, total body water, i.e. the urea space, is 63% of the body weight [78]. This fraction does not change in rats as a result of diabetes [74], bile duct ligation, or, most important for the present study, of endotoxaemia [20], although these are all conditions with changed urea dynamics. Still, determination of urea accumulation is subject to some uncertainty because of the large space of distribution (the total body water) and potentially non-steady state within the pool. The fraction of urea hydrolysed in the gut and resulting ammonium recycled into urea is 10–30% in normal rats [6]. The rate of urea synthesis is therefore larger than the accumulation of urea in nephrectomised rats. The fraction of urea lost in the gut is the same in rats with diabetes [74], in bile duct-ligated rats [20], and, again highly relevant to the present study, in rats with endotoxaemia [20]. A direct measurement of urea synthesis across the liver would obviate some of these assumptions but would require access to the portal and hepatic veins, which is not possible in small animals like rats. Indeed, Hansen and Vilstrup measured the rate of urea synthesis in whole perfused rat livers and found that the urea synthesis rate was the same as that obtained with the accumulation method [79].

The rate of urea synthesis depends on amino acid concentration. The relation between blood amino acid concentration and urea synthesis can be described by barrier-limit substrate inhibition kinetics [19]. At physiological amino acid concentrations, the urea synthesis rate increases steeply with increasing amino acid concentrations. However, saturation cannot be attained because of substrate inhibition. Instead, the maximum rate of urea synthesis, i.e. the capacity of urea nitrogen synthesis (CUNS), can be determined. This maximum rate can be reached by stimulation with exogenous amino acids and determined within 95% of the theoretical Vmax. The amino acid infusion is adjusted to obtain a steady-state total blood amino-N concentration between 7.3 mmol x L-1 and 11.6 mmol x L-1, an interval in which urea synthesis approximates its maximum in rats, i.e. it is saturated and thus independent of substrate concentration. Different amino acids may lead to different amounts of N available for urea synthesis. To standardise conditions for quantitative studies on regulation of urea synthesis, alanine is therefore used as substrate. Alanine is the amino acid taken up by the liver to the largest extent [80].

Statistical methods
Within each sub-study, data were analysed by one- or two-way unpaired Student’s t-test or by the nonparametric Mann-Whitney test, as appropriate. The assumption of normality was checked using quantile-quantile plots and histograms. When comparing data from the four sub-studies conducted after TNF-α injection, data were analysed using the Kruskal-Wallis one-way analysis of variance on ranks; when significant, post-hoc tests were performed among groups by the Mann-Whitney rank-sum test. Data are presented as the mean ± SEM. Differences were considered significant when P-values were less than 0.05.

RESULTS
TNF-α studies
The results are presented as exact values in papers 1 and 2. However, when comparing all four sub-studies, results are expressed in relative values, i.e. as percentages of the mean values of the relevant control group, to compensate for inter-study differences in control values. These inter-study differences can be ascribed to environmental and seasonal changes.

TNF-α’s effect on urea synthesis
TNF-α acutely, i.e. after 3 h, increased CUNS by 40% (P = 0.03) (Figure 1).

Figure 1. Normalised capacity of urea nitrogen synthesis (CUNS). Changes in the capacity of urea nitrogen synthesis (CUNS). Results from animals injected with TNF-α are presented as relative levels compared to control animals. Bars represent mean and SEM. * indicates a significant difference (P = 0.03) compared to controls.
**Time course of urea cycle enzyme liver mRNA and protein levels and CUNS**

The time courses of urea cycle enzyme genes (Figure 2), urea cycle enzyme proteins (Figure 3), and urea synthesis (Figure 1) during TNF-α exposure were not concordant.

**Acute phase protein liver mRNA and serum levels**

The time courses of acute phase protein mRNA (Figure 4) and serum levels (Figure 5) were, however, concordant during TNF-α exposure.

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**Table 1. The urea cycle.**

Changes in urea cycle enzyme mRNA and protein levels and in the capacity of urea nitrogen synthesis (CUNS).

<table>
<thead>
<tr>
<th></th>
<th>mRNA levels</th>
<th>Protein levels</th>
<th>CUNS</th>
</tr>
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<tbody>
<tr>
<td>1 h</td>
<td>↑↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 h</td>
<td>↑↓</td>
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</tr>
<tr>
<td>24 h</td>
<td>↓↓</td>
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<td></td>
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<tr>
<td>72 h</td>
<td>↑</td>
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</table>

**Correlations between the urea cycle and acute phase proteins**

With time, we observed a progressive down-regulation of urea cycle gene expression following TNF-α administration, mirroring an up-regulation of acute phase protein mRNA levels. As expected, the latter was followed by an increase in serum levels of the acute phase proteins, whereas we observed a discrepancy between the urea cycle gene expression and the up-regulated CUNS (Figure 6).
Cytokines

The injection of TNF-α markedly increased its plasma concentration after 1 and 3 h (P < 0.001, both). At the same time, TNF-α administration increased IL-6 (P < 0.001, both) (Table 2). After 24 and 72 h, TNF-α and IL-6 levels were below the detection limit (data not shown).

Table 2. Cytokines.

Rat IL-6 and TNF-α concentrations in the controls and in animals that were injected with TNF-α. All units are in pg/ml.

<table>
<thead>
<tr>
<th></th>
<th>1 hour</th>
<th>3 hours</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TFN-α</td>
</tr>
<tr>
<td>TNF-α</td>
<td>n.d.</td>
<td>59733 ± 6910 *</td>
</tr>
<tr>
<td>IL-6</td>
<td>n.d.</td>
<td>360 ± 36</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM. n.d.: not detectable.
* P < 0.001 in comparison to the controls.

Correlations between the urea cycle and acute phase proteins

As following TNF-α administration, we observed a down-regulation of urea cycle gene expression after IL-6 injection, mirroring an up-regulation of acute phase protein mRNA levels (Figure 7).

Table 3. Hormones, blood glucose and the HOMA-index.

Normalised glucagon, corticosterone, total IGF-I, insulin, and glucose concentrations and HOMA index in controls and in animals that were injected with TNF-α. Results from animals injected with TNF-α are presented as relative levels compared to control animals.

<table>
<thead>
<tr>
<th></th>
<th>1 hour</th>
<th>3 hours</th>
<th>24 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TFN-α</td>
<td>Control</td>
<td>TFN-α</td>
</tr>
<tr>
<td>Glucagon</td>
<td>1.0 ± 0.1</td>
<td>2.4 ± 0.4 *</td>
<td>1.0 ± 0.2</td>
<td>2.4 ± 0.4 *</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>1.0 ± 0.3</td>
<td>1.9 ± 0.2 *</td>
<td>1.0 ± 0.3</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>IGF-I</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>Insulin</td>
<td>1.0 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>HOMA-index</td>
<td>1.0 ± 0.2</td>
<td>1.9 ± 0.4</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. * P < 0.05 in comparison to the controls.
IL-6 increased corticosterone, glucose, and the HOMA index (P < 0.01, all) but had no effect on glucagon, total IGF-I, or insulin (Table 4).

Cytokines

The injection of human IL-6 markedly increased its plasma concentration (P < 0.001) (Table 4), whereas plasma rat IL-6 was the same in both groups. IL-6 administration had no effect on the concentrations of TNF-α.

Table 4. Hormones, blood glucose, the HOMA index, and cytokines.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon a</td>
<td>1 (0.3; 3.5)</td>
<td>1 (0.6; 2.8)</td>
</tr>
<tr>
<td>Corticosterone a</td>
<td>27 (23; 59)</td>
<td>92 (39; 262) *</td>
</tr>
<tr>
<td>IGF-I</td>
<td>1122 ± 66</td>
<td>991 ± 50</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.77 ± 0.13</td>
<td>0.99 ± 0.12</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.0 ± 0.3</td>
<td>7.7 ± 0.2 *</td>
</tr>
<tr>
<td>HOMA index</td>
<td>5.3 ± 1.0</td>
<td>9.6 ± 1.1 *</td>
</tr>
<tr>
<td>hIL-6</td>
<td>n. d.</td>
<td>2541 ± 676 *</td>
</tr>
<tr>
<td>rIL-6</td>
<td>26.7 ± 3.7</td>
<td>33.7 ± 4.9</td>
</tr>
<tr>
<td>TNF-α</td>
<td>n. d.</td>
<td>n. d.</td>
</tr>
</tbody>
</table>

a Data are shown as medians with 95% confidence intervals. Remaining data are shown as mean ± SEM. n. d.: not detectable. * P < 0.01 in comparison to the controls.

DISCUSSION

Protocol for TNF-α administration

In the present studies, we used TNF-α to induce an acute phase response and examined the rats after four different time intervals. Administration of TNF-α is an established method for the induction of acute phase proteins in vivo [38]. TNF-α has a short half-life of about 27 min in the circulation but is able to induce an acute phase response that reaches its maximum after 24-48 h [38]. Therefore, we examined the rats after 1, 3, 24, and 72 h, 72 h representing a point when the acute phase response was expected to be completed. The size of the TNF-α dose we used was determined in a pilot study in which the animals on that dose adopted a huddled posture with pilo-erection but remained alive. The TNF-α dose was the same in all studies.

To confirm that the TNF-α dose and route of administration used were biologically effective, we examined the phosphorylation and degradation of IkB and the NF-κB protein expression; TNF-α’s effect on this pathway has previously been demonstrated [31]. These results are presented in paper 4. TNF-α potently increased the phosphorylation and degradation of IkB after 1 h. Degradation of IkB releases NF-κB and allows for NF-κB translocation into the nucleus, where it stimulates the transcription of specific genes [31]. In accordance with this, we observed changes in the liver mRNA levels of the acute phase proteins as early as 1 h after TNF-α administration; these changes were more pronounced after 3 h. After 24 h, the systemic acute phase response was fully activated, with markedly increased serum levels of the positive acute phase proteins, and signalling through NF-κB was down-regulated, as evidenced by the synthesis and restoration of IkB protein levels. These changes are in accordance with expectations based on the literature, and they support the use of our protocol for TNF-α administration [38, 81].

TNF-α up-regulated the in vivo capacity for the disposal of amino-N by urea synthesis 3 h after administration and is accordingly a possible mediator of the increased capacity for urea synthesis in active inflammation. However, no effect on CUNS was observed 1, 24, or 72 h after administration. If TNF-α had been administered by a chronic infusion technique, e.g. by implantation of osmotic pumps, we probably would have observed a more prolonged up-regulation of urea synthesis as observed in clinical inflammation with sustained elevated TNF-α levels [62]. Twenty-four hours after the administration of TNF-α, this cytokine was no longer detectable in the blood and consequently was unlikely to affect the regulation of urea synthesis per se.

Protocol for IL-6 administration

In the studies following TNF-α administration, we demonstrated that IL-6 levels increase 3 h post-treatment. Consequently, it is possible that the described effect of TNF-α on urea synthesis involves or depends on the effects of IL-6. Therefore, we examined IL-6’s effect on the regulation of urea synthesis 3 h after injection. Recombinant human IL-6 (rhIL-6) is a potent mediator of the acute phase response in rats and induces very high increases in the animals’ acute phase protein mRNA levels 3 h post-administration [28]. The stimulation of mRNA synthesis depends on the dose of injected rhIL-6. In the present study, we found a marked increase in mRNA levels, confirming that the human IL-6 and dosage that we used were biologically effective. It is, however, difficult to compare the TNF-induced IL-6 concentration in plasma with the concentration attained after rhIL-6 injection, and the derived extent of stimulation or inhibition of transcription of specific genes. IL-6 activates a transcription factor different from the ones TNF-α activates, and accordingly, we found no changes in the NF-κB signalling in the liver after IL-6 administration (data not shown). We did not measure the activity of IL-6’s intracellular signalling pathways in the present study.

Relation between regulation of urea synthesis and the acute phase proteins

We explored the activity of the urea cycle enzyme and acute phase protein genes through measurement of mRNA levels in liver tissue, which reflect gene regulation. We found a reciprocal time course of urea cycle enzyme and acute phase protein mRNA levels during TNF-α exposure; the urea cycle enzyme mRNAs were down-regulated as the acute phase protein mRNAs were up-regulated, reaching the lowest and highest levels, respectively, 24 h after TNF-α administration. The same tendency was observed during the early IL-6-induced acute phase response. Thus, at the gene level the hepatic traffic of N was directed towards diverting N for acute phase proteins. However, the increase 3 h after TNF-α administration and subsequent lack of change in CUNS, despite a fully established 24-h acute phase response, implies unchanged, irreversible loss of N. This situation evidently does not reflect optimum metabolic N economy in the stressful state after TNF-α administration and may be one of the mechanisms contributing to inflammatory catabolism and loss of body tissue during the acute phase response.

Relation between gene and in vivo regulation of urea synthesis

As regards the mechanism of the acute increase in CUNS 3 h after TNF-α administration, it is noteworthy that the expression of all urea cycle enzyme genes decreased. This was an unexpected
finding, and it differs from most of our other studies. Usually, in vivo regulation of urea synthesis occurs in parallel with changes in the relative abundance of urea gene mRNAs, suggesting that the regulation involves gene events on the transcription level [20, 82]. The finding gains credibility by the finding in our earlier study that the acute phase response was induced by LPS [21], and that there was possibly the same discrepancy between in vivo and gene regulation. Later, we extended our studies by including data on the protein expression of the urea cycle enzymes carbamoyl phosphate synthetase (CPS) and ornithine transcarbamylase (OTC) and found unchanged urea cycle enzyme protein levels. Hence, the increase in CUNS was not induced by an increased utilisation of the gene products for the synthesis of CPS and OTC enzyme proteins. Instead, the early up-regulation 3 h after TNF-α might be induced by hormonal effects on the activity of the urea cycle enzyme proteins, i.e. an activation of CPS through increased synthesis of its activator N-acetyl-glutamate. Also, a potential increase in ASS protein levels could induce the up-regulation of CUNS; in this substrate-saturated situation, the V_{max} of ASS determines the capacity of the urea cycle. In fact, TNF-α initially, i.e. after 1 h, did increase the liver mRNA levels of ASS. Unfortunately, we did not measure enzyme activity or protein levels of ASS in the present study. Thus, the mechanism responsible for the increase in CUNS was not demonstrated.

TNF-α injection further decreased urea cycle enzyme mRNA levels after 24 h, whereas CUNS and urea cycle enzyme proteins remained unchanged. This decrease in mRNAs has previously been described after LPS administration by another group, and in that study the urea cycle enzyme levels also remained unchanged after 24 h, probably due to the enzymes’ long half-life of 4–9 days [61]. Whether this decrease in urea cycle enzyme mRNA and preservation of the enzyme function is a distinct regulatory mechanism of the acute phase response is not known.

**Loss of body N**

TNF-α induces loss of body N in two ways: it induces proteolysis in muscles and acts on liver function by increasing the in vivo capacity of urea synthesis. Accordingly, a diversion of amino acids from muscles to liver is induced, leading to negative N balance and N excess. Some of these amino acids are used for synthesis of acute phase proteins, but because the amino acid composition of acute phase proteins is different from that of the degraded muscle proteins, there is some net N excess [83]. Urea excretion is increased during an acute phase response [84] due to this increased supply of amino-N, but also due to the up-regulation of in vivo urea synthesis found in the present and previous studies [21, 62]. It is not possible to judge the fraction of loss of body N that depends on the hepatic component of inflammatory catabolism. This requires simultaneous measurements of concentration gradients and flow across the liver and muscle protein synthesis and breakdown during inflammation. Such data were not available in the present study. Because of the short investigational period, 3 h, the time interval after which CUNS is up-regulated, it was not possible to measure changes in muscle protein.

**Mediators of urea synthesis regulation**

CUNS was only affected 3 h after TNF-α administration. At that time, glucagon and IL-6 were also increased. Glucagon is known to be a powerful stimulator of urea synthesis and is of primary importance in many catabolic states; glucagon is regarded as one of the potential mediators of “the hepatic catabolic stress response” [63]. However, the hormone has no acute effect on the capacity of this process in rats [13] and thus probably plays no role for the present results. In addition, IL-6 has no effect on CUNS 3 h post-administration of TNF-α, as demonstrated in our IL-6 study. No change in other well-known regulators of urea synthesis was observed. Therefore, TNF-α is very likely a direct mediator of the up-regulation of urea synthesis observed in active inflammation.

On the genetic level, there are several possible mediators of the regulation of urea cycle enzymes during inflammation. We found that both TNF-α and IL-6 administration induced a down-regulation of the expression of the genes. Because TNF-α injection increased circulating IL-6 levels and plasma TNF-α levels were below our detection limit after IL-6 administration, it is likely that IL-6 is the direct mediator of the down-regulation of urea cycle genes. TNF-α and IL-6 do not activate the same intracellular signalling pathways, and therefore it is plausible that they have different intracellular effects regarding the regulation of urea synthesis. Also, IGF-I has previously been reported to decrease the gene expression of urea cycle enzymes [17], and in the study conducted 24 h after TNF-α administration, we actually observed increased circulating levels of IGF-I. However, inflammation is reported to reduce total IGF-I [85, 86], and accordingly, IGF-I is hardly the mediator of the down-regulation of the gene expression during inflammation in general. Insulin is another down-regulator of urea synthesis [87], and 24 h after TNF-α administration, a tendency towards increased insulin levels was observed. However, 72 h after administration of TNF-α, the urea cycle enzyme gene levels had normalised at a time when insulin levels were significantly increased. Therefore, insulin in itself probably plays no major regulatory role with regard to the decrease in gene expression.

**CONCLUSIONS**

- TNF-α administration in rats has a post-transcriptional up-regulating effect on the in vivo capacity of urea synthesis 3 h after administration, whereas IL-6, while playing a role at the genetic level during the early acute phase response, did not acutely change CUNS.

- Changes in the expression of urea cycle enzyme genes did not parallel changes in CUNS: mRNA levels of the urea cycle enzymes were predominantly decreased despite an unchanged or up-regulated CUNS.

- Changes in the protein levels of the urea cycle enzymes CPS and OTC paralleled changes in CUNS, whereas a dissociation of the effects of TNF-α on the urea cycle enzymes and on CUNS was observed 3 h post-treatment.

- A lack of mutual N stoichiometric adjustment between protein synthesis and urea synthesis during the TNF-α-induced acute phase response was observed. The lack of down-regulation of whole body urea synthesis may promote the loss of N from the body and contribute towards inflammatory catabolism.

**FUTURE PERSPECTIVES**

Our aim was to achieve a more profound understanding of the regulation of urea synthesis during inflammation compared to our previous studies; however, many questions are still left to be answered.

**Further studies on the regulatory mechanisms of urea synthesis during an acute phase response**
The up-regulation of CUNS may be induced by changes in urea cycle enzyme activity, and therefore, it would be highly relevant to conduct studies whose aim is to examine these activities, most pertinent the activity of the flux-generating enzyme CPS and the rate-limiting enzyme ASS.

Another likely explanation for the up-regulation of CUNS is increased ASS protein levels. Accordingly, it would be of great interest to extend our Western blot analyses of liver tissue by using ASS antibodies.

To demonstrate a potential connection and/or regulatory mechanism between N elimination via urea synthesis and the acute phase response after TNF-α exposure, it would be essential to conduct a more thorough investigation of intracellular signalling pathways, of the transcription of genetic information, and of potential post-translational modifications. In this way, a relation to the up-regulation of CUNS might be elucidated.

Studies on the regulation of urea synthesis in different animal models

The present study is an acute study conducted after a single injection of a pro-inflammatory cytokine, inducing an acute phase response. It would be of interest to study the regulation of urea synthesis during a type of inflammation that is more physiological than the one we studied, i.e. in an animal inflammatory model such as experimental colitis or steatohepatitis.

Liver impairment affects the regulation of urea synthesis. Previously, we studied the effect of LPS on the regulation of urea synthesis in rats with experimental cirrhosis. Furthermore, it would be highly relevant to study such regulation in experimental models of partial hepatectomy.

Clinical perspectives

The recognition of the hepatic contribution to inflammatory catabolism provides a rationale for new strategies. By a blockade of the response it may be possible to prevent or modify this hepatic contribution and improve the recovery of patients with inflammatory conditions (active inflammation). In the treatment of patients with active inflammatory bowels disease (IBD), antibodies against TNF-α are widely used, but the effect on N homeostasis of biological therapy is unknown. Urea synthesis is up-regulated in patients with active IBD [62]. It would be interesting to extend these studies with examinations of the effect of medical treatment; e.g. TNF-α antibodies and an unspecific anti-inflammatory drug like prednisolone, on the regulation of urea synthesis during active inflammation. Also, in the treatment of alcoholic hepatitis, an antibody against TNF-α is used, viz. pentoxifylline. Likewise, investigations on the regulation of urea synthesis in these patients would be of great interest.

SUMMARY

Catabolism is a serious clinical problem in patients with active inflammation. Under such stressful conditions, the catabolism and loss of tissue nitrogen (N) result from proteinolysis and are augmented by an up-regulation of the hepatic capacity to eliminate amino-N via urea-N. Our earlier studies suggest that this is part of the acute phase response to inflammation despite the increased need for amino-N for incorporation into acute phase proteins synthesised by the liver. It is, therefore, pathophysiologically and potentially therapeutically important to identify regulators of urea synthesis which, in this way, aggravate the inflammatory loss of body N; this study aimed at identifying such mediators, quantifying their effects, and unravelling their mode of action. The cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) play key roles in inflammation, and they both induce protein breakdown and catabolism. Therefore, they are both potential mediators of the up-regulation of urea synthesis.

Our first experiments showed that TNF-α administration in rats acutely, i.e. after 3 h, up-regulated the in vivo capacity of urea-N synthesis (CUNS) by 30%, whereas IL-6 was observed not to acutely change CUNS.

Furthermore, our experiments aimed at characterising the regulation of hepatic N elimination via urea during different phases of the TNF-α-induced acute phase response and to identify the steps between gene expression and physiological function that might be involved. We did so by using four different methods 1, 3, 24, and 72 h after TNF-α injection in rats: examination of urea cycle enzyme mRNA levels in liver tissue, the hepatocyte urea cycle enzyme proteins, CUNS, and known hormonal regulators of CUNS. The major serum acute phase proteins and their liver mRNA levels were also measured. Despite a progressive down-regulation of the urea cycle genes and a fully established acute phase response 24 h after TNF-α administration, no change in the in vivo capacity for the disposal of amino-N by urea synthesis was observed 1, 24, and 72 h after TNF-α injection. Moreover, TNF-α actually up-regulated urea synthesis 3 h after administration (cf. above). The dissociation of the effects of TNF-α on the urea genes and on physiological functions remains unexplained. The lack of down-regulation of whole body urea synthesis may promote the loss of N from the body and contribute towards inflammatory catabolism. This indicates the presence of an independent hepatic component of the inflammation response that is of primary importance for the stress-catabolic state.

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