Regulation of urea synthesis during the acute-phase response in rats

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CATABOLISM IS A SERIOUS CLINICAL problem in patients with acute inflammation and an acute-phase response (8, 20). The loss of body nitrogen (N), ultimately by elimination via urine urea-N, presents a threat to the integrity of the organism. Insight into the interplay between hepatic synthesis and release of acute-phase proteins and ureagenesis is important for the mechanistic understanding of the dynamics of catabolic N conversion during inflammation and for potential intervention.

During an acute-phase response there is a marked increase in hepatic export protein synthesis. In that situation, therefore, it would be advantageous to the whole-body N economy for the liver to produce less urea relative to the prevailing amino-N concentration because nitrogen incorporated into urea is irrevocably lost to the organism. This would imply downregulation of the gene expression of urea cycle enzymes, mirroring the upregulation of the mRNA levels of acute-phase proteins and, stoichiometrically more important, a simultaneous decrease in the in vivo capacity of urea synthesis (CUNS). However, our previous studies have shown that clinical or experimentally induced active inflammation rather upregulates CUNS, which promotes N loss from the body (9, 15, 23). This leads to the critical catabolism of the acute-phase response in which amino-N is wasted via increased urea synthesis despite the increased need for availability of amino-N for the buildup of acute-phase proteins. This phenomenon has not been mechanistically explored.

Therefore, we pursued this line of thought by means of studies on an experimental acute-phase response induced in rats by tumor necrosis factor-α (TNF-α). TNF-α is a central direct mediator of the response (4) and an inducer of protein breakdown and catabolism (2, 26). TNF-α was previously found to upregulate CUNS in rats 3 h posttreatment (23). However, at the same time there was a progressive downregulation of urea cycle enzyme gene expression (23, 25). To examine this discrepancy the present work aimed at extending the previous data with detailed time course experiments on the regulation of urea synthesis during TNF exposure using four methods. We examined the urea cycle enzyme mRNA levels in liver tissue, the hepatocyte urea cycle enzyme proteins, the in vivo capacity of urea-N synthesis (CUNS), and known humoral regulators of CUNS at 1, 3, 24, and 72 h after TNF-α injection.

Cytokines; inflammation; metabolism; rats; tumor necrosis factor-α; urea

MATERIALS AND METHODS

Design. The study consisted of four substudies conducted 1, 3, 24, and 72 h after injection of either placebo (vehicle saline 0.9%) or TNF-α. Some of the data from the studies conducted 3 and 24 h after the TNF-α injection were included in earlier publications (23, 25).

Animals. Female Wistar rats (body weight 200–210 g; Taconic M&B, Ejby, Denmark) were housed at 21 ± 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 (Altromin, Lage, Germany). Following anesthesia via the inhalation of 2–3% isoflurane (Forene, Abbott Laboratories, Genfoffe, Denmark), 0.2 ml of saline or 25 μg/kg of recombinant rat TNF-α (rTNF-α TriChem ApS, Frederikssund, Denmark; catalog no. 400-14) that had been dissolved in 0.2 ml of isotonic saline was intravenously injected into the tail vein of each rat. Following the injections, cage-to-cage pair feeding of animals was instituted; the control animals were given the same amount of food as that consumed by the TNF-α injected actually did stimulate intracellular signaling via the NF-κB pathway.
In each substudy, the control group consisted of 18–24 animals, and the intervention group consisted of 20–26 animals. In 8–12 animals of the control group and 10–14 animals of the intervention group in each study, the blood α-amino N concentration and the blood urea concentration were measured, and CUNS was determined. In the remaining 10–12 animals of each group, the following variables were measured: protein levels of NF-xB, total IkB and phosphorylated IkB in liver tissue, protein expression and mRNA levels of the urea cycle enzymes carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), and argininosuccinate synthetase (ASS) in liver tissue; mRNA levels of the urea cycle enzymes argininosuccinate lyase (ASL) and arginase (ARG) in liver tissue; liver mRNA levels of the prevailing rat acute-phase proteins α-2-macroglobulin (α2MG), haptoglobin, α-1-acid glycoprotein (α1AGP), albumin, and thiostatin; plasma concentrations of glucagon, corticosterone, glucose, and insulin; serum concentrations of the acute-phase proteins α2MG, haptoglobin, α1AGP, and albumin; and serum concentrations of the cytokines TNF-α and interleukin 6 (IL-6).

CUNS. One, 3, and 72 h after the injection of TNF-α or saline and following anesthesia with a subcutaneous injection of fentanyl/fluansione (Hynporm, Jansen Pharma, Birkened, Denmark) at 0.5 ml/kg and midazolam (Dormicular, Roche, Basel, Switzerland) at 2.5 mg/kg, retroperitoneal nephrectomy was performed, a catheter was inserted into the femoral vein such that alanine could be infused, and CUNS was determined as previously described (23). Because the control animals’ CUNS levels varied in the four independent experiments, the CUNS results from animals injected with TNF-α are presented as relative values compared with control animals within each experiment.

Liver tissue. After anesthesia was administered, ~200 mg of liver tissue from the left lobe was harvested, snap-frozen in liquid N2, and stored at ~80°C. Aliquots of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Bio-Rad Criterion Gel System (Bio-Rad, Hercules, CA). The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, which were then blocked with 2% nonfat dried milk (Sigma-Aldrich, Brondby; Denmark) in Tris-buffered saline Tween-20 (10 mM Tris, 150 mM NaCl, pH 7.8, and 0.1% Tween-20). The blocked membranes were incubated with antibodies against CPS 1, OTC, ASS 1 (Abcam, Cambridge, UK), NF-xB, IkB, and phospho-IkB (all from Cell Signaling, Beverly, MA). The membranes were washed and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Amersham, Pharmacia Biotech, Piscataway, NJ) as a secondary antibody, and the proteins were visualized by BioWest enhanced chemiluminescence (UVP, Upland, CA) and quantified by use of a UVP BioImaging System (UVP). mRNA levels of urea enzymes and acute-phase proteins were determined by slot blot hybridization as previously described (15, 16).

Blood analyses. Blood urea was measured by the urease-Berthelot method (1), whereas the total blood α-amino-nitrogen level was measured via the dinitrofluorobenzene method (3). CUNS was calculated as the body accumulation of urea corrected for intestinal hydrolysis, as previously described (6). The concentrations of the prevailing rat acute-phase proteins α2MG, haptoglobin, α1AGP, and albumin in serum were evaluated via specific rat ELISAs (Immunology Consultants Laboratory, Newberg, OR; Life Diagnostics, West Chester, PA; and Alpha Diagnostic, San Antonio, TX). Samples were assayed in duplicate. All assays had intra- and interassay coefficients of variance that were below 5% and 10%, respectively. Plasma glucagon was measured by wick chromatography in the studies conducted 3 and 24 h after TNF-α injection (18). In the studies conducted 1 and 72 h after TNF-α injection, plasma glucagon was measured by RIA. Glucagon immunoreactivity was measured via the C terminally directed antiserum 4305, which only detects glucagon of pancreatic origin, with highly purified porcine glucagon as a control (17). Plasma corticosterone concentrations were assessed by use of a specific rat RIA kit (Amersham Biosciences, Buckinghamshire, UK). The serum insulin level was measured by an ultrasensitive rat insulin ELISA (DRG Diagnostics, Marburg, Germany), and the plasma glucose level was determined by a routine analytical method. The HOMA index was calculated in accordance with a previously described model (10). The plasma IL-6 level was determined by use of xMAP technology (Luminex Corporate, Austin, TX). In the studies at 3 and 24 h, the plasma TNF-α level was determined by use of xMAP technology (Luminex Corporate, Austin, TX). All of the assays that were used in this study have been validated for use in rats.

Statistical methods. Each substudy was conducted as an independent study with its own control group. Within each substudy data were analyzed by the unpaired Mann-Whitney rank-sum test. The data of the time course changes were analyzed by the Kruskal-Wallis one-way analysis of variance on ranks. Data are presented as means ± SE. Differences were considered significant when P values were less than 0.05.

RESULTS

Urea cycle enzyme liver mRNA and protein levels and CUNS. The time courses of urea cycle enzyme mRNA levels, urea cycle enzyme protein levels, and urea synthesis during TNF-α exposure were not concordant. TNF-α had no effect on the liver protein levels of the urea cycle enzymes CPS, OTC, or ASS at any time (Fig. 1A). However, ASS1 has a predicted molecular weight of 47 kDa and, as shown in Fig. 1B, ASS protein degradation products were clearly detectable 1 and 3 h after TNF-α injections as by Western blotting as lower molecular weight bands. This was associated with increased mRNA levels of ASS by (11%) and ARG by (15%) 1 h after administration (Fig. 1, B and C). At 3 h after administration, the mRNA levels of all of the urea cycle enzyme genes started to decrease; 24 h after administration, TNF-α decreased the liver mRNA level of the CPS gene by 51 ± 3%, the OTC gene by 28 ± 3%, the ASS gene by 48 ± 4%, the ASL gene by 61 ± 4%, and the ARG gene by 37 ± 4% (P < 0.001, all). By 72 h after TNF-α administration, the levels had normalized (Fig. 1C). CUNS increased by 40% (P = 0.03) 3 h after injection compared with the controls (Fig. 1D). When analyzing the time course of normalized CUNS there was the same trend in CUNS following TNF-α injection, though not significant at the 5% level (P = 0.07, Kruskal-Wallis ANOVA).

Acute-phase protein liver mRNA and serum levels. The time courses of acute-phase protein mRNA and serum levels were concordant. TNF-α increased the liver mRNA levels of α2MG and haptoglobin after 3 h, with further increases of 257 ± 43% and 195 ± 6%, respectively (P < 0.001, both) 24 h after administration. TNF-α increased the liver mRNA levels of α1AGP as early as 1 h after administration; after 24 h, α1AGP increased by 459 ± 13% (P < 0.001). Liver mRNA levels of thiostatin initially decreased but started to increase after 3 h and reached the highest levels at 24 h, with a 163 ± 6% increase compared with the controls (P < 0.001). The albumin liver mRNA levels started to decrease after 1 h and decreased by an additional 14 ± 5% (P = 0.05) after 24 h. By 72 h after TNF-α administration, all the acute-phase protein mRNAs tended to normalize to baseline (Fig. 2A).

In the circulation, TNF-α increased α2MG 30-fold, haptoglobin 5-fold, and α1AGP 8-fold only after 24 h (P < 0.01, all). After 72 h, the serum acute-phase proteins were still increased but to a lesser extent. TNF-α decreased serum
Fig. 1. Normalized urea enzyme protein expression (A), argininosuccinate synthetase (ASS) mRNA and protein expression (B), urea cycle enzyme mRNA levels (C) and capacity of urea nitrogen synthesis (CUNS) (D). Changes in the protein expression of carbamoyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), and ASS assessed by Western blotting analyses (A); mRNA levels and protein expression of ASS (B); mRNA levels of CPS, OTC, ASS, argininosuccinate lyase (ASL) and arginase (ARG) in liver tissue assessed by slot blotting analyses (selected data shown) (C); and changes in the CUNS (D). Results from animals injected with TNF-α are presented as relative levels compared with control animals. Bars represent means and SE. *Significant difference (P < 0.05) compared with controls.
albumin after 24 h, with a further decrease to 69% after 72 h (P < 0.001) (Fig. 2).

Correlations between the urea cycle and acute-phase proteins. With time after TNF-α administration, there was a progressive downregulation of urea cycle gene mRNAs and an opposite upregulation of acute-phase protein mRNAs. The latter was followed by an increase in the serum levels of the acute-phase proteins, whereas there was a dissociation between the downregulated urea cycle gene expression and the upregulated CUNS (Fig. 3).

Plasma glucagon, corticosterone, insulin, and glucose and the HOMA-index. At 1 h after administration, TNF-α increased glucagon (P < 0.001) and corticosterone (P = 0.01), whereas only glucagon was increased after 3 h (P < 0.01). TNF-α had no effect on glucagon or corticosterone 24 and 72 h after administration. Glucose levels remained unchanged at all times. There was a trend toward increased insulin levels after 1 (P = 0.07) and 24 h (P = 0.14); after 72 h, a statistically significantly increase was observed (P = 0.04). Likewise, the HOMA-index was unchanged after 1, 3, and 24 h and increased after 72 h (P = 0.03) (Table 1).

Cytokines. The injection of TNF-α markedly increased its plasma concentration after 1 [59,733 ± 6,910 pg/ml vs. not detectable (n.d.)] and 3 h (81 ± 31 pg/ml vs. n.d.) (P < 0.001, both). At the same time, TNF-α administration increased IL-6 levels [360 ± 36 (1 h) and 284 ± 44 pg/ml (3 h) vs. n.d.; P < 0.001, both]. After 24 and 72 h, both TNF-α and IL-6 were below the detection limit (data not shown).

Activation of NF-κB signaling in the liver. TNF-α increased the phosphorylation of IκB (inhibitor of NF-κB) by approximately sixfold and approximately twofold after 1 and 3 h, respectively (P < 0.01, both), associated with an ~80% decrease in IκB content after 1 h (P < 0.01) and an ~10% increase after 24 and 72 h (P < 0.05, both) (Fig. 4). NF-κB protein expression was not affected by TNF-α administration.

DISCUSSION

A central finding of this study was the reciprocal time courses of urea cycle enzyme and acute-phase protein mRNA levels following TNF-α exposure: the urea cycle mRNAs were...
downregulated at the same time as the acute-phase protein mRNAs were upregulated. Despite the downregulation of the urea cycle genes, there was no decrease in the in vivo capacity for the disposal of amino-N by urea, and TNF-α actually upregulated urea synthesis 3 h after administration. This lack of downregulation of urea synthesis may aggravate the loss of nitrogen from the body and contribute to dangerous inflammatory catabolism. In contrast, the increases in acute-phase protein gene expression were directly reflected in increases in the serum levels of these proteins.

In this study, we used TNF-α to induce an acute-phase response and examined the rats after four different time intervals. The TNF-α dose was the same in all studies. The administration of TNF-α potently increased the phosphorylation and degradation of IkB after 1 h, which confirms that the protocol for TNF-α administration we used was biologically effective. Degradation of IkB releases NF-κB and allows for NF-κB translocation into the nucleus, where it stimulates the transcription of specific genes (21) and accordingly the liver mRNA levels of the acute-phase proteins rose as early as 1 h after TNF-α administration. This was more evident after 3 h, and after 24 h, the complete systemic acute-phase response was activated. The signaling through NF-κB was then downregulated, as seen from the synthesis and restoration of IkB protein levels. These changes are in accordance with expectations based on the literature (4, 12).

We used four methods to study the dynamics of the regulatory steps on different levels of urea synthesis. On the gene level, we explored the expression of the urea cycle enzyme genes through measurement of mRNA levels in liver tissue. We found that the mRNA levels were decreased by TNF-α reaching the lowest levels 24 h after TNF-α administration. On the posttranslational level, we explored the expression of hepatocyte urea enzyme proteins. These proteins showed no change over time after TNF-α. On the whole-body physiological level, we measured the capacity of urea synthesis (CUNS) during a saturating intravenous infusion of alanine, ensuring that changes reflected substrate-independent regulation. CUNS was increased 3 h after TNF-α administration but was no different from controls 1, 24, or 72 h after TNF-α. Finally, on the level of humoral regulation, we measured known circulating regulators of CUNS. We found only minor changes in “stress hormones,” insulin, and glucose, with no expected effect on urea synthesis.

Most previous studies on the regulation of urea synthesis in a variety of physiological and pathophysiological settings show that changes in the expression of urea cycle enzyme genes and changes in CUNS parallel one another (5, 14) as would be expectable from the assumption of a downstream sequential manifestation of gene events. However, in the present study, the mRNA levels of the urea cycle enzymes were decreased and still the urea cycle enzyme proteins were unchanged and CUNS likewise unchanged or even increased. This disruption of the usual sequence remains unexplained but may be related to changes, i.e., increases, in utilization of the gene products resulting from the translation of the genes. An alternative explanation that we suggested in an earlier publication is a decrease in the enzyme synthesis rate, due to the decreased gene activity, and simultaneously an even more pronounced decrease in the degradation of the enzyme, i.e., a decreased turnover of the enzyme (23). However, this explanation would not fit with the findings in the present paper, viz. that the urea cycle enzyme proteins were unchanged.

The steps of the urea cycle at which urea synthesis may be controlled and regulated are mainly catalyzed by the urea cycle enzymes CPS and ASS (22). CPS is the feeder enzyme that determines the flux through the urea cycle, and ASS is the ultimate rate-limiting enzyme because it has the lowest in vitro V_max among the urea cycle enzymes. The CUNS method measures the substrate-saturated capacity of urea synthesis, attained by offering the urea cycle an abundance of substrate. This stimulates the CPS through increased synthesis of its allosteric activator N-acetyl-glutamate and does not require increased levels of the CPS enzyme protein to increase flux through the cycle (11). In this saturated situation, the capacity of the urea cycle is probably determined by the V_max of the ASS. Therefore, an increase in the activity or amount of ASS could give rise to the increase in CUNS 3 h after TNF-α. We did not directly measure the catalytic activity of ASS but we found initially increased liver mRNA levels of ASS, unchanged ASS protein expression, and at the same time increased expression of degradation products from ASS. This increased turnover of proteins indicates an increased activity of ASS and may explain the upregulated CUNS despite the decreased gene activity.

Initially, TNF-α increased the liver mRNA levels of ARG at a time when CUNS was not yet upregulated. ARG is neither flux nor rate controlling for urea synthesis but is known to be an inflammatory marker that is released from hepatocytes in response to an inflammatory stimulus (13). Thus the dynamics of ARG is rather a marker of liver injury than of regulation of urea synthesis.

In vivo, the hepatic urea synthesis rate is determined by the blood concentrations of α-amino nitrogen, and various hor-
mones and cytokines regulate this substrate-product relationship. We found that the glucagon levels were increased when CUNS was upregulated, i.e., 3 h after TNF-α. Glucagon, despite being a powerful stimulator of the actual rate of urea synthesis, has no short-term effect on the capacity of the process and thus likely plays no role in the phenomena described here (19). TNF-α also increased circulating IL-6 after 1 and 3 h. However, we have earlier shown that IL-6 does not change CUNS (24).

We found a progressive downregulation of urea cycle gene expression with time after TNF-α administration, opposite the upregulation of acute-phase protein mRNA levels. Still, the whole-body CUNS rose and subsequently maintained control values in face of the fully established acute-phase response after 24 h. This implies unchanged irreversible loss of nitrogen to urea throughout the acute-phase response. This situation evidently does not reflect optimum metabolic nitrogen economy in the stressful acute-phase state and is probably one of the mechanisms contributing toward inflammatory catabolism and loss of body tissue.

In conclusion, there was no decrease in the in vivo capacity of urea synthesis despite a fully established acute-phase response. At the gene level, however, the hepatic traffic of nitrogen seemed to be directed so as to divert nitrogen for acute-phase proteins; still, this nitrogen-conserving gene adjustment was overruled and thus the in vivo urea synthesis was not orchestrated together with acute-phase protein synthesis to the effect of limiting N waste. This deterioration of whole-body nitrogen economy may become a threat to the integrity of the organism in patients with various states of active inflammation. The mechanism of the disturbed interplay between gene activity and physiological function remains unknown. The background for the resulting seemingly inappropriateness of nitrogen economy should probably be sought in evolutionary events. One possibility is that the acute-phase response relies on an abundance of amino acids, leaving a need for elimination of toxic ammonia and that maintenance of this possibility has been evolutionarily advantageous. Another possibility is that the phenomenon is rooted in remnant functions from the diverse earlier evolutionary roles of urea (27), where urea first in the last part of the evolution plays the role of a nitrogen conversion regulator. In parallel, we have earlier shown in humans that the phylogenetically old function of urea as an osmoregulator still overrules the role of urea as a nitrogen scavenger (7).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


