Modulation of tumor necrosis factor-α release by anisoosmolarity and betaine in rat liver macrophages (Kupffer cells)

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Abstract Hypoosmotic exposure (205 mosmol/l) of rat liver macrophages together with lipopolysaccharide (LPS) inhibited the LPS-induced tumor necrosis factor-α (TNF-α) release by about 60% and markedly diminished the LPS-induced increase of TNF-α mRNA levels. Hyperosmotic exposure (405 mosmol/l) had no effect on total TNF-α release, however, both TNF-α accumulation in the medium and the LPS-induced increase of TNF-α mRNA levels were significantly delayed under these conditions. This delay was abolished upon addition of betaine, which acts as an osmolyte in Kupffer cells. When LPS was added to Kupffer cells that had been preexposed to hyperosmotic medium for 24 h, the LPS-induced TNF-α release was inhibited by 90% when compared to normoosmotic conditions. Likewise, the LPS-induced increase in TNF-α mRNA levels was largely abolished. Inhibition of TNF-α release and of the increase in the TNF-α mRNA level in response to hyperosmolarity/LPS, however, was largely overcome when indomethacin or betaine was present during the hyperosmotic preincubation period. Because betaine has recently been shown to inhibit the hyperosmolarity-induced induction of cyclooxygenase-2 and stimulation of prostaglandin production, these findings suggest that the effect of betaine in restoring the LPS-induced TNF-α response in hyperosmotically exposed Kupffer cells is mediated by an inhibition of prostaglandin synthesis. The findings point to a regulatory role of cell volume and betaine for TNF-α production by liver macrophages, suggesting a new role of osmolytes in modulating immune function.

Key words: Kupffer cell; Betaine; Osmolyte; Hyperosmolarity; Cell volume; Osmolarity; Tumor necrosis factor; Macrophage; BGT-1

1. Introduction

Liver macrophages (Kupffer cells) belong to the mononuclear phagocyte system and play an important role in the body's defense machinery (for review see [1]). Upon stimulation with endotoxin these cells produce a variety of cytokines, lipid mediators and radicals. Recent studies have indicated that endotoxin enhances the expression of inducible cyclooxygenase-2 (Cox-2) [2–6], which results in an increased formation of prostanoids by Kupffer cells and other macrophages [3,7–9]. This response to LPS is markedly enhanced in hyperosmotic environments due to an about 10-fold stimulation of the expression of Cox-2 when ambient osmolarity increases from 300 to 350 mosmol/l [3]. This remarkably sensitive and potent osmoregulation suggested that cell volume homeostasis is critical for Kupffer cell function. Subsequent studies identified betaine as an organic osmolyte in Kupffer cells [10] and RAW 264.7 mouse macrophages [11]. The mRNA levels coding for the betaine transporter BGT-1 are strongly induced by hyperosmolarity in these cells [10,11]. Organic osmolytes are compounds which are specifically accumulated or released by the cells in response to hyperosmotic cell shrinkage or hypoosmotic cell swelling in order to maintain cell volume homeostasis (for reviews see [12–14]). The functional significance of hyperosmolarity-induced betaine accumulation in Kupffer cells is suggested by the fact that betaine was shown to suppress the hyperosmolarity-induced stimulation of prostaglandin formation and Cox-2 expression [10]. Thus, osmolyte availability and BGT-1 expression are another site of regulation of Kupffer cell function. The present study was undertaken in order to get insight into the role of anisoosmolarity and betaine in the regulation of tumor necrosis factor-α (TNF-α) formation in rat Kupffer cells. The data show that lipopolysaccharide-induced TNF formation is strongly affected by anisotonicity and betaine and point to a role of betaine in the modulation of the immune response by Kupffer cells.

2. Materials and methods

2.1. Materials

RPMI 1640 medium (without phenol red) and fetal calf serum (FCS) for culture of Kupffer cells were from Biochrom (Berlin, Germany). RNeasy total RNA kit was from Qiagen (Hilden, Germany) and oligonucleotide labelling kit was from Pharmica (Freiburg, Germany). Sodium dodecyl sulfate was from Fluka (Karlsruhe, Germany). Nycodenz was from Nycomed (Oslo, Norway). Hybond-N nylon membranes were purchased from Amersham Buchler (Braunschweig, Germany). [α-^{32}P]dCTP (3000 Ci/mmol) was from ICN (Meckenheim, Germany). Betaine and lipopolysaccharide (LPS) were from Sigma (Deisenhofen, Germany). Recombinant mouse TNF-α and hamster anti-rMuTNF mAbs were from Genzyme (Rüsselsheim, Germany). A plasmid containing rat TNF-α cDNA [15] was a gift from Dr. K. Decker (Freiburg, Germany). The 1.0 kb cDNA fragment for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used for standardization, was from Clontech (Palo Alto, USA).

2.2. Isolation and culture of Kupffer cells

Rat Kupffer cells were prepared by collagenase-pronase perfusion and separated by a single Nycodenz gradient and centrifugal elutriation as described previously [3,16]. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS for 48 h. The experiments were performed during the following 24 h using Krebs-Henseleit hydrogen carbonate buffer (pH 7.4) containing 10 mmol/l glucose. The osmolarity of the medium was varied by changing the NaCl concentration. The viability of Kupffer cells was more than 95% as assessed by trypan blue exclusion. Viability of the incubations was

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Abbreviations: Anti-rMu-TNF-α mAb, anti-mouse recombinant tumor necrosis factor-α monoclonal antibody; Cox-1, cyclooxygenase-1 (constitutive); Cox-2, cyclooxygenase-2 (inducible); LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α

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routinely tested by lactate dehydrogenase (LDH) release at the end of the incubations.

2.3. Tumor necrosis factor-α assays

Enzyme linked immunoassay. TNF-α was quantitated by a double-sandwich enzyme-linked immunosorbent assay (ELISA) as described previously [17]. Briefly, 200 ng of hamster anti-rMuTNF-α mAb was bound to the surface of 96-well plates by incubation for 18 h at 4°C in 0.1 mol/l carbonate buffer (pH 9.6). Uncoupled binding sites in the wells were blocked with 0.1 mol/l carbonate buffer containing 1% defatted milk. Wells were incubated with 100 μl of a sample twice overnight at 4°C. Plates were washed with 0.02 mol/l PBS containing 0.05% Tween 20 and then exposed to 5 μg of rabbit polyclonal anti-rMuTNF-α in PBS. The plate was developed using peroxidase-labeled goat anti-rabbit Ig and ABTS. rMuTNF-α was used as an assay standard.

Bioassay. TNF-α content of the supernatants of Kupffer cells was assayed by determining the cytotoxicity of the supernatants following addition to TNF-α-sensitive L929 cells [18]. Briefly, L929 cells were plated in 96-well plates at a density of 4×10^5/well. The culture medium to be tested was added to the well together with 400 ng actinomycin D per well. Cell survival was quantified by light microscopy as well as by measuring the uptake of crystal violet in an ELISA reader. The test was standardized with recombinant mouse TNF-α.

2.4. Northern blot analysis

Total RNA from near-confluent culture plates of Kupffer cells was isolated using RNeasy Total RNA Kit (Qiagen). RNA samples were electrophoresed in 0.8% agarose/3% formaldehyde and then blotted onto Hybond-N nylon membranes with 20×SSC (3 mol/l NaCl, 0.3 mol/l sodium citrate). After a brief rinse with water and UV-cross-linking (Hoefer UV-crosslinker 500, Hoefer, San Francisco, USA), the membranes were observed under UV illumination to determine RNA integrity and location of the 28S and 18S rRNA bands. Blots were then subjected to a 3-h prehybridization at 43°C in 50% deionized formamide, in sodium phosphate buffer (0.25 mol/l, pH 7.2), containing 0.25 mol/l NaCl, 1 mmol/l EDTA, 100 μg/ml salmon sperm DNA and 7% SDS. Hybridization was carried out in the same solution with approx. 10^6 cpm/ml [α-^{32}P]dCTP-labeled random primed TNF-α or GAPDH cDNA probes. Membranes were washed three times in 2×SSC/0.1% SDS for 10 min, twice in sodium phosphate buffer (25 mmol/l, pH 7.2)/EDTA (1 mmol/l)/0.1% SDS and twice in sodium phosphate buffer (25 mmol/l, pH 7.2)/EDTA (1 mmol/l)/1% SDS. Blots were then exposed to Kodak AR X-omat film at −70°C with intensifying screens. Suitably exposed autoradiograms were then analyzed with densitometry scanning (PDI, New York, USA).

2.5. Statistics

Values are expressed as means ± S.E.M. (n=number of cell preparations). Statistical analysis was performed using Student's t-test. $P<0.05$ was considered to be statistically significant.

3. Results

In line with previous data (for a review see [1]), TNF-α production by cultured rat liver macrophages (Kupffer cells) was stimulated upon addition of LPS in normoosmotic (305 mosmol/l) incubations; within 3–4 h after LPS addition maximal TNF-α concentrations were obtained in the incubations. When LPS was added together with institution of hypoosmotic (205 mosmol/l) conditions, TNF-α accumulation occurred with a similar time course, however, the maximal TNF-α concentration reached was only about 40% of that recovered in normoosmotic incubations. On the other hand, institution of hyperosmotic (405 mosmol/l) conditions together with LPS produced maximal TNF-α levels comparable to those found in normoosmotic incubations, however, TNF-α accumulated much slower under these conditions and maximum levels were reached after about 8 h (Fig. 1). This hyperosmolarity-induced delay in TNF-α release was largely abolished when betaine (10 mmol/l) was added (Fig. 2). The osmolarity-dependent differences in LPS-induced TNF-α production, as detected by the TNF-ELISA technique, were also confirmed in the TNF-α cytotoxicity assay (data not shown), indicating that the effects of osmolarity on TNF protein were parallelled by effects on the bioactivity of TNF-α.

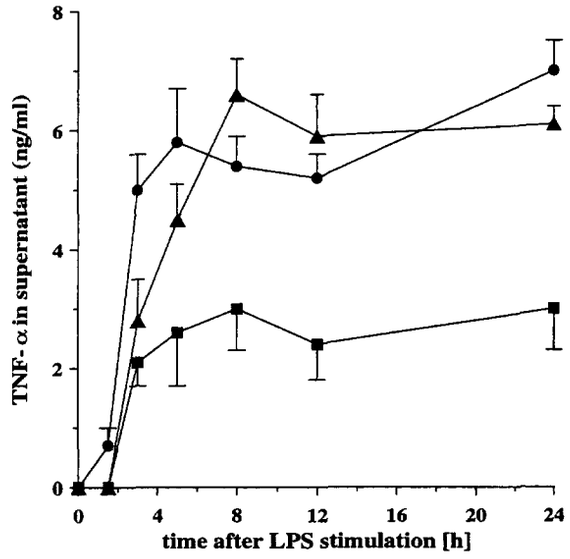

Fig. 1. Effect of anisoosmolarity on the LPS-stimulated tumor necrosis factor-α (TNF-α) production in Kupffer cells. Rat Kupffer cells were kept for 48 h in normoosmotic (305 mosmol/l) medium and then ($t=0$) exposed to LPS (0.5 μg/ml)-containing media at osmolarities of 205 (■), 305 (●) or 405 (▲) mosmol/l. TNF-α in the supernatant was measured by ELISA. Data are given as means ± S.E.M. ($n=4$).

The effects of osmolarity on LPS-induced TNF-α formation (Figs. 1 and 2) were also reflected in the time-dependent increase of TNF-α mRNA levels. Anisoosmotic exposure by itself had no effect on TNF-α mRNA levels within 1.5 h (Fig. 3). As further shown in Fig. 3, addition of LPS to normoosmotically exposed Kupffer cells led to a significant induction of TNF mRNA within 90 min, which became maximal after 3–4 h and rapidly declined thereafter (Fig. 3). When, however, LPS was added to the hypoosmotic (205 mosmol/l) stimulus, the increase in TNF-α mRNA levels was strongly blunted in line with the diminished TNF-α formation depicted in Fig. 1. On the other hand, LPS together with hyperosmotic stress led to a strong increase of TNF-α mRNA which, however, was delayed when compared to the normoosmotic control. Addition of betaine augmented the LPS-induced rise in TNF-α mRNA levels in hyperosmotic incubations, resulting in a time course similar to that observed under normoosmotic conditions (Fig. 3). These findings suggest that ambient osmolarity and betaine are important modulators of LPS-induced TNF-α formation in Kupffer cells.

When Kupffer cells were exposed for 24 h to anisoosmotic media before a 3-h stimulation period with LPS, hypoosmotic exposure had little effect on TNF-α production, whereas hyperosmotic exposure inhibited TNF-α production by more than 90% (Table 1). This was also observed at the TNF-α mRNA level: whereas hypoosmotic exposure had no effect on the LPS-stimulated TNF-α mRNA induction, TNF-α mRNA was almost undetectable when LPS was added to hyperosmotically preexposed Kupffer cells (Fig. 4). However, both TNF-α mRNA levels (Fig. 4) and TNF-α production

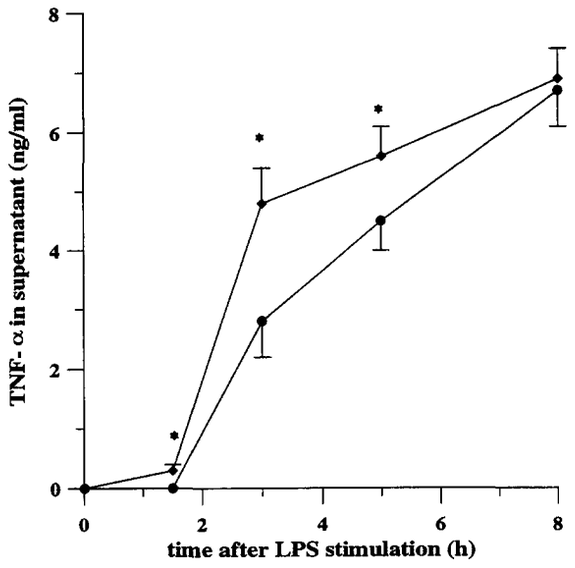

Fig. 2. Effect of betaine on LPS-stimulated TNF-α production in hyperosmotically exposed rat Kupffer cells. Rat Kupffer cells were exposed for 12 h to normoosmotic (305 mosmol/l) medium in the absence or presence of betaine (10 mmol/l). Then ($t = 0$), LPS (0.5 μg/ml) was added and the osmolarity of the media was changed to 405 mosmol/l (● = without betaine; ◆ = with betaine). TNF-α in the supernatant was measured by ELISA after the time period indicated. Data are given as means ± S.E.M. ($n = 4$). *Significantly different from the hyperosmotic (405 mosmol/l) condition without betaine ($P < 0.05$).

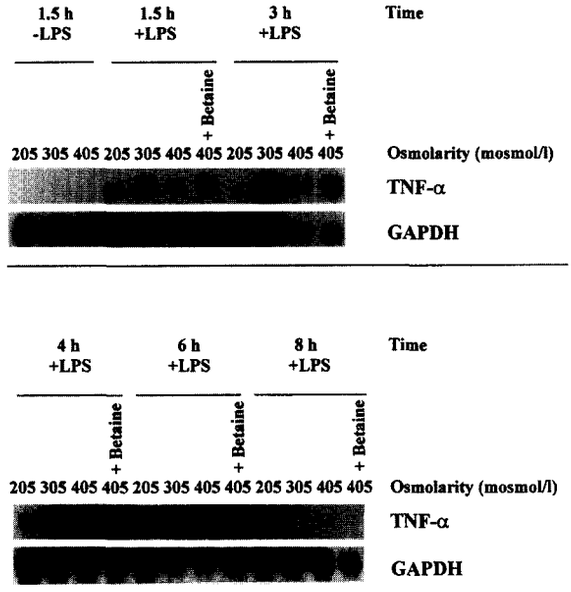

Fig. 3. Effect of anisoosmolarity and betaine on the LPS-induced increase of TNF-α mRNA levels in rat Kupffer cells. Rat Kupffer cells were cultured for 48 h and then exposed to hypoosmotic (205 mosmol/l), normoosmotic (305 mosmol/l) or hyperosmotic (405 mosmol/l) medium in the absence or presence of LPS (0.5 μg/ml) and/or betaine (10 mmol/l) for the time period indicated. Thereafter the cells were harvested for RNA isolation and subjected to Northern blot analysis for TNF-α and GAPDH (7 μg of total RNA per lane).

(Table 1) in response to LPS were markedly increased close to normoosmotic levels, when the hyperosmotic medium contained betaine (10 mmol/l) or indomethacin (10 μmol/l) (Fig. 4, Table 1).

4. Discussion

TNF-α is mainly produced by macrophages and blood monocytes in response to inflammatory stimuli, such as LPS and some viruses (for review see [1,19]). In endotoxin shock Kupffer cells are a major source of this cytokine, which is believed to mediate most endotoxin effects in vivo. TNF-α synthesis is inhibited by glucocorticoids and prostaglandin E_2 [20–22] and regulation takes place at the transcriptional and translational level. In the present study, ambient osmolarity was identified as another factor modulating the TNF-α production by Kupffer cells in response to LPS. Acute hypoosmotic exposure together with LPS strongly reduced TNF-α production, whereas hyperosmolarity delayed the appearance of the cytokine, when compared to normoosmotic control conditions (Fig. 1). TNF mRNA levels roughly parallelled the formation of immunoreactive TNF-α (Fig. 3). The mechanism by which anisoosmolarity affects LPS-induced TNF expression remains unclear. However, both LPS signal transduction in macrophages [23–25] and osmosignalling in other cell types [26,27] involve an activation of mitogen-activated protein (MAP) kinases. Thus, one may speculate about an interaction between LPS action and osmolarity at the level of signal transduction.

Interestingly, betaine was able to abolish the hyperosmolarity-induced delay in TNF-α formation (Figs. 2 and 3), indicating that osmolytes can interfere with the immune response of these cells. The effect of betaine was even more pronounced when Kupffer cells were kept in hyperosmotic media 12–24 h before the LPS challenge. Under these conditions the presence of this osmolyte restored LPS-induced TNF-α production and mRNA levels to the normoosmotic level, whereas in its absence LPS was no longer able to induce a TNF-α response

Table 1
Effect of long-term (24 h) anisoosmotic exposure on LPS-stimulated TNF-α release

	TNF-α (ng/ml)	Inhibition (%)	LDH release (U/l)
205 mosmol/l	5.7 ± 0.6	−18	5.7 ± 0.6
305 mosmol/l	4.9 ± 0.4	0	5.9 ± 0.6
405 mosmol/l	0.3 ± 0.2	94	6.3 ± 0.8
405 mosmol/l+betaine	4.0 ± 0.4	17	6.0 ± 0.8
405 mosmol/l+indomethacin	3.7 ± 0.4	25	6.1 ± 0.7

Rat Kupffer cells were incubated for 24 h in hypoosmotic (205 mosmol/l), normoosmotic (305 mosmol/l) or hyperosmotic (405 mosmol/l) medium in the absence or presence of betaine (10 mmol/l) or indomethacin (10 μmol/l). The osmolarity was changed by appropriate changes of the NaCl concentration. Thereafter LPS (0.5 μg/ml) was added and the amount of TNF-α produced during the following 3 h was measured by ELISA. Lactate dehydrogenase (LDH) release was measured at the end of the incubations. Data are given as means ± S.E.M. ($n = 5$).

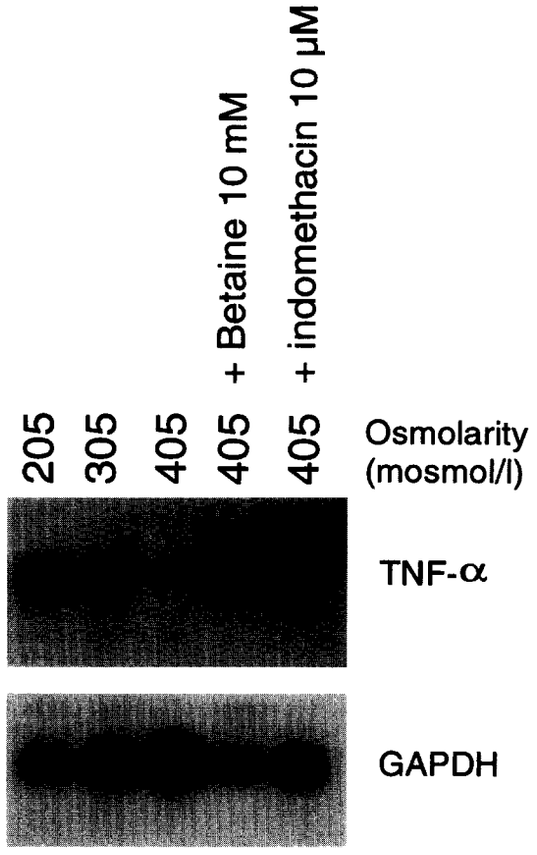

Fig. 4. Effect of anisoosmotic preexposure, betaine and indomethacin on TNF-α mRNA levels following addition of LPS in rat Kupffer cells. Rat Kupffer cells were cultured for 48 h and then exposed to media with the osmolarities indicated and supplemented with either betaine (10 mmol/l) or indomethacin (10 μmol/l) for 24 h. Then LPS (0.5 μg/ml) was added for 1.5 h. Thereafter the cells were harvested for RNA isolation and subjected to Northern blot analysis for TNF-α and GAPDH (7 μg of total RNA per lane).

(Table 1, Fig. 4). This effect of betaine probably resides at the level of eicosanoid formation. Prolonged hyperosmotic exposure was recently shown to enhance LPS-induced Cox-2 expression and prostaglandin E_2 (PGE_2) formation up to 10-fold and some induction of Cox-2 and stimulation of PGE_2 formation was even observed in the absence of added LPS during strong hyperosmotic (405 mosmol/l) stress (see Figs. 1, 5 and 6 in [3]). Since PGE_2 is known to suppress TNF-α expression [20], it is highly likely that enhanced PGE_2 levels during hyperosmolarity explain the abolition of the TNF response when LPS is added. In line with this suggestion, not only indomethacin, but also betaine, which was shown to abolish the hyperosmolarity-induced Cox-2 expression and PGE_2 formation [10], restored the LPS sensitivity of TNF-α formation.

The pathophysiological relevance of the present findings remains to be established. However, plasma hypo- and hyperosmolarities are frequently found in critically ill patients, which may influence their immunological condition. Here the new perspective arises on a role of osmolytes during immune responses.

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