Hypoxia enhances lysosomal TNF-α degradation in mouse peritoneal macrophages

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Hypoxia enhances lysosomal TNF-α degradation in mouse peritoneal macrophages. Am J Physiol Cell Physiol 295: C2–C12, 2008. First published April 23, 2008; doi:10.1152/ajpcell.00572.2007.—Infection, simulated by lipopolysaccharide (LPS), is a potent stimulator of tumor necrosis factor-α (TNF-α) production, and hypoxia often synergizes with LPS to induce higher levels of the secreted cytokine. However, we show that in primary mouse peritoneal macrophages and in three mouse peritoneal macrophage cell lines (RAW 264.7, J774A.1, and PMJ-2R), hypoxia (O2 < 0.3%) reduces the secretion of LPS-induced TNF-α (P < 0.01). In RAW 264.7 cells this reduction was not regulated transcriptionally as TNF-α mRNA levels remained unchanged. Rather, hypoxia and LPS reduced the intracellular levels of TNF-α by twofold (P < 0.01) by enhancing its degradation in the lysosomes and inhibiting its secretion via secretory lysosomes, as shown by confocal microscopy and verified by the use of the lysosome inhibitor Bafilomycin A1. In addition, although hypoxia did not change the accumulation of the soluble receptor TNF-R1, it increased its binding to the secreted TNF-α by twofold (P < 0.05). We suggest that these two posttranslational regulatory checkpoints coexist in hypoxia and may partially explain the reduced secretion and diminished biological activity of TNF-α in hypoxic peritoneal macrophages.

cytokines; inflammation; trafficking; secretory lysosomes

HYPOXIA IS A CENTRAL FEATURE of ischemic, inflamed, and infected tissues and a principal determinant of the pathophysiology of local and generalized systemic inflammatory responses (SIR) in these conditions (27). Infection, increased oxygen consumption by proliferating bacteria and inflammatory/immune cells, as well as hemodynamic alterations, are some of the proposed mechanisms that underlie this phenomenon (27). Among all causes of septic SIR, intraperitoneal infection is a relatively common clinical entity that frequently occurs in chronic liver diseases and complicates abdominal trauma, operations, and bowel perforation. The latter are usually caused by a mixed bacterial population and have been shown to cause peritoneal hypoxia (35). Hence, both exposures to hypoxia and bacterial proinflammatory mediators are important components in the pathophysiology of local and systemic inflammatory responses (28), in clinical sepsis in general, and in infectious peritonitis in particular. Tumor necrosis factor-α (TNF-α) secreted mostly by macrophages plays a central role in mediating excessive inflammatory responses in these conditions (13, 33). Since hypoxia is a major constituent of ischemia and LPS is a potent transcriptional and translational inducer of TNF-α (5, 48) and a key mediator of SIR, their combined effect is commonly employed to mimic a clinically relevant in vivo scenario of inflammatory response in ischemia and infection. So far a number of studies have shown that hypoxia synergizes with LPS to enhance the production and release of TNF-α from human, rat, and mouse monocytes and macrophages (7, 16, 21, 26, 36, 46). This synergism was mainly attributed to transcriptional upregulation involving nuclear factor (NF)κB (7, 24), although other mechanisms such as reduction in cAMP were also suggested (26). However, hypoxia has also been demonstrated to inhibit or not to change the amounts of secreted LPS-induced TNF-α (29, 46). The reduction was explained by enhanced sensitivity of these macrophages to hypoxia-mediated apoptosis. Furthermore, the effects of circulating or secreted TNF-α may also be regulated by its binding to the soluble forms of the two TNF receptors, sTNFR-I and sTNFR-II, which can bind and inhibit its activity (4). Indeed, elevated levels of these receptors have been found in patients with different pathologies, whose common denominator is hypoxia (8, 12, 42), and hypoxia was shown to increase their secretion in vitro (36).

In this study we evaluated changes in the secretion and activity of TNF-α from thioglycollate (TG)-elicited primary peritoneal macrophages and the mouse peritoneal macrophage cell lines RAW 264.7, PMJ-2R, and J774A.1 following exposure to LPS and hypoxia, where LPS represents gram-negative infection or ischemia-associated bacterial translocation, and hypoxia is characteristic of an inflammatory microenvironment or simulates ischemia. In our model of simulated hypoxia/infection in peritoneal macrophages, we show that hypoxia reduced the secretion and biological activity of TNF-α without associated cell death and suggest that at least two posttranslational mechanisms, enhanced lysosomal degradation of TNF-α, and increased extracellular binding to soluble TNF receptor II are responsible for this phenomenon.

MATERIALS AND METHODS

Cells. Primary peritoneal macrophages, isolated from both female and male BALB/c and C57Bl/6 mice, were collected 4 days after an intraperitoneal injection of 3 ml of 24 mg/ml solution TG, by injecting their peritoneal cavity with 10 ml of phosphate-buffered saline (PBS), massaging, and then drawing back to the syringe the fluid now containing the macrophages. Washed TG-elicited peritoneal macrophages were plated at a concentration of 0.8 × 10⁶ cells/well for 2 h

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in Dulbecco’s modified Eagle’s medium (DMEM) and 20% fetal calf serum (FCS) to facilitate adherence, following replacement of the medium with DMEM without FCS (serum starvation) for 1 h before their exposure to the experimental conditions to avoid possible masking of signals initiated by the exogenous stimuli in the FCS. Purity of all the adherent primary macrophages was routinely ≥84 ± 2.9% as was evaluated by labeling the cells with FITC-conjugated rat anti-mouse F4/80 (Serotec, Oxford, UK) and analyzing its surface expression by flow cytometry. This part of the study was performed in adherence to the National Institutes of Health Guide For The Care and Use of Laboratory Animals. The mouse peritoneal macrophage cell lines RAW 264.7, J774.A1 (derived from BALB/c mice), and PMJ2R (derived from C57BL/6j mice) were cultured in DMEM with 10% FCS and antibiotics and were similarly serum-starved 1 h before the beginning of the experiment. All cells were subjected to normoxia or hypoxia for 24 h, with or without the addition of LPS (1 μg/ml, Escherichia coli 055:B5, Sigma, St. Louis, MO). In some experiments cells were incubated with increasing amounts of bafilomycin A1 (Calbiochem, Darmstadt, Germany) and MG132 (Calbiochem), which did not cause cell death, as assessed by the 2.3-bis-(2-methoxy-4-nitro-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) kit. In all experiments cell viability was determined using the XTT kit (Biological industries, Beit-Haemek, Israel).

Normoxic and hypoxic conditions. For normoxic conditions cells were incubated in a regular incubator (21% O2-5%-CO2-74% N2). Hypoxic incubation was performed in a sealed anaerobic workstation (Concept 400, Ruskin Technologies, Leeds, UK), where the hypoxic environment (O2 < 0.3%, 5% CO2-95% N2), the temperature (37°C), and humidity (90%) were kept constant. Samples from the culture medium were taken at the end of the incubation period to measure the partial pressures of O2 and CO2, as well as pH, using a blood gas analyzer ABL510 (Radiometer, Denmark). Mean PO2 values in the supernatants were 146 ± 19 mmHg in normoxia and 26 ± 0.9 mmHg in hypoxia, whereas the mean values of PCO2 (32.5 ± 0.3 mmHg) and the pH values (7.4 ± 0.07) did not change in normoxia and hypoxia. ELISA. Commercial TNF-α, soluble TNF receptor I, and soluble TNF receptor II ELISA kits (R&D Systems, Minneapolis, MN) were carried out according to the manufacturer’s instructions. TfN biological activity. TNF-α secretion from RAW 264.7 cells was determined by its biological activity as well as by an ELISA assay. To measure the biological activity, L-929 fibroblast cells at a concentration of 5 × 104 cells/well were plated in 96-well flat bottom plates. When the monolayer became confluent, 4 μg/ml actinomycin D (Sigma) together with either the mouse recombinant TNF-α or the experimental supernatants were added. After 24 h incubation, XTT (30 μl/well) was added and incubated for 4 h at 37°C. The absorbance at 450 nm (reference of 620 nm) was then read, and measurements for the dilution series were plotted to produce dose-response curves, in which the OD read was proportional to the reciprocal of the concentration of biologically active TNF-α.

Quantitative real-time PCR analyses. Total RNA was extracted from 4 × 106 RAW 264.7 cells exposed to the experimental conditions using TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Five micrograms of total RNA were transcribed to cDNA at 37°C for 1 h using 200 μM deoxynucleotides (Sigma), 5 μM random hexamers (Amersham Pharmacia Biotech, Piscataway, NJ), 20 U RNAsguard (Amersham), and 200 U/μl of MMLV-RT (US Biochemicals, Cleveland, OH). TNF-α mRNA expression was determined by quantitative real-time PCR on the cDNA samples using the TaqMan assay on demand kit with the ABI-PRISM 7000 (Applied Biosystems, Foster City, CA). Analysis was carried out in triplicates in a volume of 20 μl (2 min at 50°C, 10 min at 95°C, and a total of 40 cycles, each of 15 s at 95°C and 1 min at 60°C) for both TNF-α and the endogenous reference gene 18S rRNA, which does not change in hypoxia, and the comparative threshold cycle (Ct) method was used. In each experiment the normoxic nonstimulated RNA sample was used as a calibrator to allow comparison of relative quantity between the samples. Western blots analyses. RAW 264.7 cells were lysed in RIPA buffer, their supernatants were collected and concentrated 30-fold by VivaSpin2 (Vivascience, Lincoln, UK), and equal protein amounts or equal volumes were loaded on a 10% SDS-PAGE. Proteins were separated and transferred onto cellulose nitrate membranes (Schleicher and Schuell). The membranes were incubated for 1 h in blocking buffer (20% skimmed milk, 1% BSA, 0.01% Tween-20, 10 mM Tris pH 8.0, and 150 mM NaCl) at room temperature, probed for 1 h with the dilution of 1:250 rabbit polyclonal anti-TNF-α (Genzyme, Cambridge, MA), washed three times in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.5% Tween-20), and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:5,000 for additional 1 h. The enhanced chemiluminescence system (Amersham) was used for detection. The optical density of the bands was quantified using Bio-Imaging system (Dinco & Rhenium, Jerusalem, Israel) and TINA software (Raytest, Straubenhardt Germany).

Immunoprecipitation. RAW 264.7 cells were cultured and exposed to normoxia or hypoxia, with or without the addition of LPS for 24 h. Cells were harvested in RIPA buffer and protein concentrations of the cellular extracts were measured by Bradford reagent. To avoid nonspecific binding, 200 μg of protein were incubated with 2 μg of normal mouse serum and precipitated using protein-A agarose beads. The remaining proteins were incubated with 2 μg of hamster polyclonal anti-mouse TNFR1 or TNFR-II (R&D systems) and rotated overnight at 4°C with protein-A agarose. After centrifugation the pellet was washed four times in PBS, resuspended in loading buffer, boiled for 15 min, and loaded onto a 10% SDS-PAGE. After electrophoretic separation, TNF-α was detected by Western blot analysis as described before.

Flow cytometry analysis. RAW 264.7 cells were cultured in normoxia or hypoxia for 24 h, with or without the addition of LPS, and then were labeled with goat anti-mouse TNF-α (R&D systems) and FITC-conjugated donkey anti-goat (R&D systems). After washing was completed, the cells were fixed in 0.1% formaldehyde and were analyzed using a Cytometer-XL flow cytometer (Coulter Electronics). Dead cells were excluded from the analysis by their forward and side light-scattering properties.

Immunofluorescence. RAW 264.7 cells were incubated in the experimental conditions on coverslips, fixed with 3.7% formaldehyde for 10 min at room temperature, and permeabilized in 0.1% Triton X-100. To avoid nonspecific binding the cells were incubated with 4% donkey normal serum for 30 min at room temperature following three washes with PBS. The primary antibodies goat anti-mouse TNF-α (R&D systems) or rabbit polyclonal anti-Rab8, anti-Rab5, and anti-Rab7, anti-Rab11, anti-Rab4, and anti-LAMP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated for 1 h at room temperature following three washes with PBS. Secondary antibodies (Rhodamine Red-X or Cy3-conjugated donkey anti-goat IgG or Cy2-conjugated donkey anti-rabbit IgG, Jackson ImmunoResearch Laboratories, West Grove, PA) were incubated for 1 h at room temperature following three washes with PBS. The coverslips were mounted on a slide with fluoromount G. Immunofluorescent images were acquired by confocal microscopy, using the Bio-Rad MRC 1000 confocal system, and the images were analyzed using the ImagePro Plus 4.5 software (Media Cybernetics, Silver Spring, MD).

Statistical analyses. All values are presented as means ± SE. The data were analyzed using repeated measures analysis of variance (ANOVA). The Student-Newman-Keuls multiple comparisons test was used to evaluate significance between experimental groups, and P values exceeding 0.05 were not considered significant.
RESULTS

Hypoxia reduces LPS-induced secretion of TNF-α. In primary peritoneal macrophages, incubation in normoxia or hypoxia for 24 or 48 h was not sufficient to induce TNF-α secretion, and stimulation with LPS was required. In normoxia, LPS induced TNF-α secretion, but although hypoxia did not induce cell death, as evaluated by the XTT viability test (data not shown), it reduced this secretion by 2.5-fold (P < 0.01) after 24 h (Fig. 1A). A similar effect persisted after 48 h of incubation (11,265 ± 3,012 pg/ml in normoxia and 5,664 ± 981 pg/ml in hypoxia, P < 0.01). Of note, analysis of these results showed a twofold difference in the magnitude of TNF-α secretion between male- and female-derived TG-elicited peritoneal macrophages incubated in normoxia and LPS (13,253 ± 3,367 in males vs. 26,877 ± 8,822 pg/ml in females, P < 0.01); however, hypoxia similarly reduced the levels of the cytokine by 2- and 2.8-fold, respectively (P < 0.05 and P < 0.001). Similarly, incubation of the mouse peritoneal macrophage cell line RAW 264.7 in normoxia and hypoxia, with or without LPS did not cause cell death after 24 h (Fig. 1B) or 48 h (data not shown). In the absence of LPS, TNF-α secretion was hardly detected by ELISA (Fig. 1C), whereas addition of LPS for 24 and 48 h induced it (92,970 ± 18,024 and 87,450 ± 30,050 pg/ml, respectively, P < 0.001). In contrast, incubation in hypoxia resulted in a significant threefold reduction in TNF-α secretion after 24 h (P < 0.001) and 48 h (P < 0.05) of incubation. This inhibitory effect of hypoxia on RAW 264.7 cells was validated by a biological activity assay (3.9-fold, P < 0.001, Fig. 1D) and by Western blot analysis (2.7-fold, P < 0.05, Fig. 1E). The difference in TNF-α concentrations measured in the ELISA and biological assays may reflect the essential difference between the techniques. ELISA also revealed that two other peritoneal macrophage cell lines, J774.1 (Fig. 1F) and PMJ2R, also exhibited a similar 2.8-fold and 1.5-fold reduction in LPS-induced secretion of TNF-α in hypoxia (2,815 ± 772 pg/ml and 1,005 ± 664 pg/ml, P < 0.05 for J774A.1 and 10,810 ± 721 pg/ml in normoxia vs. 7,238 ± 333 pg/ml in hypoxia, in PMJ2R, P < 0.01). Of note, hypoxia, even with LPS, did not cause any cell death in these two cell lines (0.96 ± 0.05- and 1.02 ± 0.02-fold from control, for J774.1 and PMJ2R, respectively). Although concentrations of TNF-α were different in each cell line, probably due to
differences in strains and/or gender, the three peritoneal macrophage cell lines and the primary peritoneal macrophages exhibited similar reduction in TNF-α secretion in hypoxia. We focused on the RAW 264.7 cell line to further study the mechanistic explanations of this phenomenon.

Hypoxia does not change the steady-state levels of TNF-α mRNA in RAW 264.7 cells. To evaluate the effects of hypoxia on transcription of TNF-α, we used real-time PCR to measure the relative accumulation of TNF-α mRNA following stimulation with LPS in both normoxia and hypoxia. Each sample was normalized to the endogenous reference 18S ribosomal RNA, which does not change in hypoxia, as do other housekeeping genes such as β-actin or GAPDH (49). Whereas hypoxia alone increased TNF-α mRNA by 1.7 ± 0.2-fold, this tendency did not reach statistical significance relative to the normoxic nonstimulated cells (Fig. 2A). LPS significantly upregulated the accumulation of TNF-α mRNA (by 4.9 ± 0.7-fold, \( P < 0.001 \)) in macrophages in normoxia, and these levels did not change in hypoxia. Moreover, the increase in TNF-α mRNA levels following LPS stimulation cannot explain the thousandfold increment in TNF secretion. These observations do not support transcriptional regulation as an important mechanism controlling the hypoxia-induced suppression of TNF-α secretion and directed our attention to posttranscriptional mechanisms involved in the regulation of TNF-α production and secretion.

Hypoxia does not cause intracellular accumulation of TNF-α or disrupt its trafficking. In nonstimulated cells, intracellular concentrations of TNF-α protein measured by Western blot analysis and ELISA were very low (Fig. 2, B and C), but addition of LPS in normoxia increased it by 13- and 11-fold, respectively (\( P < 0.001 \)). However, in hypoxia the intracellular concentrations of TNF-α were significantly reduced by 2.5-fold in Western blot analysis (\( P < 0.05 \)) and by 2-fold in ELISA (\( P < 0.01 \)) when compared with the stimulated normoxic values, corresponding to the hypoxia-induced inhibition of TNF-α secretion and suggesting that TNF-α protein is not accumulating in hypoxic cells. Furthermore, since TNF-α is cleaved off the membrane by TNF-α converting enzyme (TACE), we also checked its expression by FACS. Nonstimulated cells in normoxia expressed low levels of TACE, and LPS was required to enhance its expression by threefold (mean fluorescence of 0.67 ± 0.11 and 2.22 ± 0.4, respectively, \( P < 0.05 \)). Hypoxia alone or hypoxia with LPS did not significantly change TACE expression (2.7 ± 0.6 and 1.92 ± 0.8, respectively).

Using confocal microscopy, we further evaluated TNF-α trafficking in the cells and its colocalization with several Rab proteins that characterize transport vesicles that could be implicated in TNF-α trafficking: Rab8, which is a marker of secretory vesicles and vesicles mediating polarized delivery of membrane proteins; Rab5, which is a marker of early endosomes; Rab4 and Rab11, which are markers of recycling endosomes that are associated with turnover of membranal proteins; Rab7, which is a marker of late endosomes linking transport between them to the lysosomes and back; LAMP-1, which is a marker of lysosomes; and Rab27a, which is a marker of secretory lysosomes that mediate secretion of some proteins in cells of the immune system. Isotype-matched control sera yielded no immunostaining or only a very weak staining. TNF-α was not induced in nonstimulated cells, whereas the staining of all Rab proteins was constitutive and was not affected by hypoxia (data not shown). Analysis of the images demonstrated that in hypoxia the mean area of the cells was significantly decreased by 1.6-fold (206 ± 13 μm² compared with 337 ± 31 μm² in normoxia, \( P < 0.01 \)). To correct for changes in the fluorescent signal that may occur due to the decreased area, we used the integrated optical density (IOD), which takes into account the area of the cells. The IOD values for each fluorescently labeled protein was calculated separately and determined in three replications of the experiment, in at least five different fields. Staining for Rab7 and for Rab8 did not change in hypoxia and was not colocalized with TNF-α in either normoxia or hypoxia (data not shown), suggesting that...
secretory vesicles and late endosomes do not carry TNF-α.

Intracellular TNF-α was decreased by 33% in hypoxia compared with normoxia (P < 0.001), confirming our previous results (Fig. 3, B and H, Fig. 4, B and E, H and K, M and Q), whereas staining for Rab5 (Fig. 3, A and G), Rab4 (Fig. 4, G and J), Rab11 (data not shown), Rab27a (Fig. 4, A and D), and LAMP-1 (Fig. 4, N and P) did not significantly change in hypoxia. In both normoxia and hypoxia TNF-α was found colocalized to Rab5 (Fig. 3, C and I), Rab4 (Fig. 4, I and L), and Rab27a (Fig. 4, O and R), but in hypoxia this colocalization was reduced. In contrast, colocalization of TNF-α with LAMP-1 was increased by 84% in hypoxia (P < 0.05).

Hypoxia increases TNF-α degradation in the lysosome, involving Rab5-expressing endosomes. Hypoxia-induced degradation of TNF-α was investigated by using the proteosome inhibitor MG132 and the lysosome inhibitor bafilomycin A1. Intracellular or secreted amounts of TNF-α were not affected by inhibition of the proteosome degradation activity (Fig. 5, B and D) and remained significantly lower in hypoxia (P < 0.05 from normoxia), regardless of the increasing concentrations of

Fig. 3. Effects of hypoxia on the colocalization of TNF-α with Rab5. RAW 264.7 cells (10⁶) were plated and incubated as before. Cells were immunostained with anti-TNF-α (red, B, E, H, K) and anti-Rab5 (green, A, D, G, J). The merged TNF-α and Rab images from A+B, D+E, G+H, J+K are shown (C, F, I, L), and areas of colocalization are in orange. Magnification was ×1,600. These experiments were repeated three times, and images of at least 5 different fields were taken for analysis. TNF-α was found colocalized to Rab5 in normoxia, indicating its presence in early endosomes, whereas hypoxia inhibited this colocalization, probably as a result of decreased levels of intracellular TNF-α. Addition of bafilomycin A1 (10 nM) restored intracellular TNF-α to normoxic values.
Fig. 4. Effects of hypoxia on the colocalization of TNF-α with Rab4 and Rab27a proteins. RAW 264.7 cells (10⁶) were plated and incubated as before. Cells were immunostained with anti-TNF-α (red, B, E, H, K) and anti-Rab27a (green, A, D) or anti-Rab4 (green, G, J). The merged TNF-α and Rab images from A+B, D+E, G+H, J+K are shown (C, F, I, L), and areas of colocalization are in orange. Magnification was ×1,600. These experiments were repeated three times, and images of at least 5 different fields were taken for analysis. TNF-α was found colocalized to Rab27a and Rab4 in normoxia, indicating its presence in secretory lysosomes and in recycling endosomes, and hypoxia inhibited this colocalization, probably as a result of decreased levels of intracellular TNF-α.
MG132. In contrast, bafilomycin A1 that did not affect the intracellular levels of TNF-α in normoxic macrophages significantly increased it in hypoxic macrophages in a dose-dependent manner (Fig. 5A), so that in 5 and 10 nM of bafilomycin A1, the amounts of intracellular TNF-α were equal to those in normoxia (P < 0.05 and P < 0.01 relative to hypoxic cells without bafilomycin A1). The amounts of secreted TNF-α did not change upon addition of bafilomycin A1, further supporting the retention of TNF-α in the cells (Fig. 5C). In addition to these results, which were obtained using ELISA on cellular extracts, the effects of bafilomycin A1 were also examined by confocal microscopy (Fig. 3, D–F and J–L). In normoxia, addition of the lysosome inhibitor (10 nM) did not alter TNF-α staining in LPS-stimulated cells (Fig. 3, B and E) or its colocalization with Rab5 (Fig. 3, C and F), but in hypoxia it increased the accumulation of intracellular TNF-α by 35% (P < 0.01) compared with hypoxia alone (Fig. 3, H and K), restoring it to normoxic values.

Hypoxia decreases membranal TNF-α expression. In normoxia or hypoxia, nonstimulated RAW 264.7 cells exhibited only negligible amounts of membrane-associated TNF-α as evaluated by flow cytometry (mean fluorescence < 0.009, Fig. 6B), whereas incubation with LPS in normoxia significantly increased it (mean fluorescence 0.27 ± 0.03, P < 0.001). Hypoxia significantly decreased LPS-induced membranal attachment of TNF-α by 1.5-fold (Fig. 6, A and B, mean fluorescence 0.18 ± 0.02, P < 0.01 relative to LPS-stimulated normoxic cells), excluding the possibility that augmented membranal association of TNF-α leads to the reduced amounts of TNF-α in the supernatants.

Hypoxia does not promote degradation of secreted TNF-α but increases its binding to the soluble receptor type II. We used Western blot analysis in an attempt to detect an increase in fragmented bands of TNF-α in the hypoxic cultures. However, although hypoxia reduced the secreted level of TNF-α by 2.7-fold (P < 0.05), as indicated by the autoradiograms (Fig. 1E), in accordance with the lower biological function and immunological reactivity (Fig. 1, C and D), no additional degraded bands of the cytokine were observed.

We measured the effects of hypoxia on the concentrations of the two soluble TNF-α receptors (sTNFR-I, sTNFR-II). Figure 6C shows that in normoxia, sTNFR-I was constitutively secreted from RAW 264.7 cells (110 ± 15 pg/ml) but was significantly reduced in hypoxia (54 ± 9 pg/ml, P < 0.01). Addition of LPS elevated the concentration of sTNFR-I in normoxia (230 ± 18 pg/ml); however, its levels in hypoxia remained significantly reduced (135 ± 11 pg/ml, P < 0.01). Negligible amounts of sTNFR-II were observed in normoxia and hypoxia in nonstimulated cells (Fig. 6D), and addition of LPS similarly induced them (to about 400 ± 75 pg/ml) in both normoxia and hypoxia. We then performed immunoprecipitation (IP) using the supernatants to evaluate the actual binding of secreted TNF-α to each of the soluble receptors. As shown in Fig. 6E, binding of TNF-α to sTNFR-I in normoxia or hypoxia, with or without the addition of LPS, was hardly detected. In contrast, following LPS addition, TNF-α was bound to sTNFR-II both in normoxia and hypoxia, and hypoxia significantly increased this binding by twofold (P < 0.05).

**DISCUSSION**

It has previously been shown that hypoxia alone triggers only small amounts of TNF-α, and it synergizes with LPS to induce high secretion from both primary macrophages (10, 16, 21) and monocyte/macrophage cell lines (7, 36). This synergism has previously been attributed to the activation of NFκB and reduction of cAMP levels, leading to enhanced transcription of the TNF-α gene (7, 24, 26). Interestingly, we found suppressive effect of hypoxia on LPS-induced secretion of TNF-α from mouse peritoneal macrophages and three peritoneal cell lines (RAW 264.7, J77A.1, and PMJ2R cells). This inhibitory effect of hypoxia on LPS-induced secretion of

![Fig. 5. The lysosome inhibitor bafilomycin A1 normalizes the hypoxia-reduced intracellular accumulation of TNF-α. RAW 264.7 cells (1–3 × 10⁶) were plated and incubated as before, with the addition of increasing amounts of the lysosomal inhibitor bafilomycin A1 (n = 7) (A and C) or the proteosome inhibitor MG132 (n = 5) (B and D). TNF-α concentrations were determined by ELISA in cellular extracts (A and B) or in the supernatants (C and D). *P < 0.05 and **P < 0.01 compared with LPS-stimulated cells in hypoxia. The proteosome inhibitor MG132 had no effect, whereas the lysosome inhibitor bafilomycin A1 gradually increased the accumulation of intracellular but not secreted TNF-α in hypoxia.](http://ajpcell.physiology.org/)

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TNF-α was described before in RAW 264.7 cells and interpreted as resulting from a pro-apoptotic influence of hypoxia (46). However, we did not detect increased cell death in hypoxic RAW 264.7, J774A.1, and PMJ2R cells or in the primary peritoneal macrophages. Therefore, we suggest that the reduction of TNF-α secretion was a consequence of adaptation to the hypoxic environment and did not result from decreased cell number. The contrast between the reported synergism of LPS and hypoxia and our findings may be attributed to the specific microenvironmental influences of the peritoneum, because macrophages are known for their heterogeneous functions and responses dictated by their local microenvironment in each tissue (23). Some studies report a synergism between LPS and hypoxia despite a common peritoneal origin of the macrophages. For example, Albina et al. (2) showed an increase in TNF-α secretion from resident peritoneal cells exposed to 2% O2 and interferon (IFN)γ + LPS. Likewise, the J774A.1 cell line was shown to secrete more TNF-α upon induction with LPS and 1.5% O2 (7). The differences between these studies and our results may be explained by either the differences between resident and TG-elicited peritoneal macrophages or by the different hypoxic environment as we exposed our cells to severe hypoxia (<0.3% O2). In addition, our findings are in agreement with others, who found reduction in TNF-α secretion in hypoxia (46). Thus the effects of hypoxia on macrophage TNF-α production and secretion remain controversial.

The driving force for diffusion of oxygen into the tissue is determined by its partial pressure gradient between the capillary and tissue cells and not by oxygen content. Therefore, the hypoxic oxygen tensions in our in vitro environment (26 ± 0.9 mmHg) are relevant to the environment of the injured tissue and represent a true hypoxic environment, as normal reported peritoneal oxygen tensions measured were 45–58 mmHg (6, 35). Furthermore, key functions required for clearance of infection and repair of tissue (e.g., leukocyte killing activity, necrotic tissue proteolysis, fibroblast proliferation, collagen accumulation and crosslinking, and epitheliazation) are markedly impaired at tissue oxygen tensions below 30 mmHg and almost completely stop at significantly lower values (19, 30). Macrophages adapt well to this level of hypoxia by elevating glycolytic enzymes, TGF, FGF, VEGF, and HIF-1 and reducing the expression of others (25). We have previously shown that in macrophages hypoxia and a proinflammatory stimulus reduced the surface expression of CD80 (21), the secretion of MMP-4 (34), and the inducible NO synthase (iNOS) (9). Thus the effects of hypoxia and LPS on peritoneal macrophages may not be specific for TNF-α but reflect a general mechanism designed to reduce or restrain their proinflammatory response, although the molecular mechanisms utilized to achieve this reduction may be different for each mediator molecule. This inhibitory effect of hypoxia on LPS-induced secretion of TNF-α may be relevant in physiological conditions where, for example, septic patients were reported to exhibit an anti-inflammatory response (compensatory anti-inflammatory response syndrome, CARS) rather than a proinflammatory response (SIRS) (20). Hence, there is no one
response to stimulations such as hypoxia and LPS but rather a spectrum of responses that may depend on the type of bacterium, the specific tissue involved, the degree of hypoxia, the origin of the macrophages, and the underlying condition of the patient.

To further delineate mechanisms responsible for the observed hypoxia-induced reduction of TNF-α secretion, we used the RAW 264.7 cell line, which provided us with a large number of homogeneous cell population needed for these experiments. LPS strongly enhances TNF-α transcription, but since there was no change in the steady-state amounts of TNF-α mRNA between normoxia and hypoxia, transcriptional regulation as a mechanism explaining the hypoxia-induced reduction in TNF-α levels was ruled out. We then investigated the possibility that hypoxia posttranslationally interferes with the intracellular secretory mechanisms of the already synthesized TNF-α, implying that TNF-α would accumulate in higher amounts inside hypoxic cells or on their membranes compared with normoxic cells. However, we found reduced intracellular amounts of all TNF-α forms (e.g., the mature 17-kDa form and the 26-kDa membranal form, or additional dimers or trimers) inside RAW 264.7 cells or on their membranes following incubation in hypoxia and LPS. Of note, TNF-α is known to be stored only in mast cells (32), whereas in other cells all pro-TNF-α is transported to the membrane and cleaved by TACE. Thus the minimal levels of TACE found in nonstimulated cells may account for the expression of the 26-kDa membranal form of TNF-α found in these cells (Fig. 2B). The hypoxia-induced reduction of intracellular TNF-α, together with the lack of change in TNF-α mRNA, suggests enhanced degradation of the protein. To examine the effect of hypoxia on TNF-α degradation, we used inhibitors specific for the two major protein degrading organelles: the proteasome and the lysosome. The proteasome inhibitor MG132 did not affect the amounts of intracellular TNF-α or secreted TNF-α in both normoxia and hypoxia, thus ruling out ubiquitin-mediated degradation of TNF-α following LPS stimulation. Addition of the lysosome inhibitor bafilomycin A1 did not change the amounts of secreted TNF-α but dose dependently inhibited the degradation of intracellular TNF-α only during hypoxia, suggesting that intracellular TNF-α is directed from the early endosomes to the lysosomes for enhanced degradation during hypoxia and remains in these compartments when the lysosome and regular trafficking of TNF-α is inhibited by bafilomycin A1. Bafilomycin A1 was shown to increase cytokine production in normoxic peritoneal macrophages that were not induced by LPS (17); however, only minute amounts of TNF-α were detected, suggesting that this effect was probably related to the inhibition of TNF-α recycling. Although we did not directly check the effects of hypoxia on the translation of TNF-α, the dose-dependent inhibition of TNF-α by bafilomycin A1, that at high concentrations reached normoxic values, renders this regulatory checkpoint unlikely.

Transportation of TNF-α in specialized secretory and endosomal vesicles directs it toward secretion, membranal expression, and degradation. It is now accepted that TNF-α is synthesized as a 26-kDa protein that is first accumulated in the Golgi complex (37), where early proteolytic processing into its 17 kDa mature form may occur (39). Then it is transported by the secretory machinery to the cell surface, where it is degraded by TACE and secreted as the mature protein. As part of its turnover, membranal TNF-α can be endocytosed and directed to the recycling endosome, where it is either degraded in the lysosome or directed back to the membrane (38, 43). However, the detailed pathway of TNF-α trafficking in LPS-stimulated macrophages is yet far from clear. The possibility that hypoxia affects trafficking of proteins destined for secretion has recently been introduced, with emphasis on secretory vesicles (34) and on vesicles mediating recycling of membranal proteins (45). To explore this, we used the Rab proteins, belonging to the family of Rab small GTPases that are involved in the regulation of intracellular vesicle transportation (40, 47). The Rab proteins chosen served as markers for secretory vesicles that direct vesicle transport from the Golgi complex to the cell surface (Rab8), late endosomes (Rab7), and early endosomes (Rab5), recycling endosomes (Rab4 and Rab11), lysosomes (LAMP-1), and secretory lysosomes (Rab27a). In LPS-stimulated macrophages in normoxia, TNF-α was colocalized with Rab5, Rab4, and Rab11, indicating a role for early and recycling endosomes, but not for late endosomes or secretory vesicles, in TNF-α trafficking. Furthermore, we were able to show colocalization between TNF-α and Rab27a or TNF-α and LAMP-1, suggesting a role for the lysosomes and secretory lysosomes in TNF-α secretion. In hypoxia, colocalization of TNF-α with these Rab proteins was significantly reduced, probably due to the reduced amounts of the intracellular cytokine, because staining for the Rab proteins did not change. However, colocalization with LAMP-1 was increased. The lysosome inhibitor bafilomycin A1 did not change the expression of TNF-α or its colocalization with Rab5 in normoxia, but in hypoxia it restored intracellular levels of TNF-α and its colocalization with Rab5. These observations, combined with the ability of bafilomycin A1 to dose-dependently prevent

Fig. 7. A suggested model for TNF-α trafficking. In normoxia, TNF-α is transported from the Golgi to the early endosomes (EE), and from there it reaches the membrane via the lysosome (L) and secretory lysosomes (SL). Membranal TNF-α is cleaved by TNF-α converting enzyme (TACE) and secreted or endocytosed into recycling endosomes (RE). The normoxic route is indicated in thin arrows. Hypoxia enhances degradation of TNF-α in the lysosome (indicated by the large thick arrow), causing less TNF-α to be transported to the secretory lysosomes and to the membrane. The Rab proteins that are used as specific markers for each compartment are indicated.
hypoxia-induced TNF-α lysosomal degradation, suggest that in normoxia TNF-α is transported from the early endosome via the lysosome to the secretory lysosomes, and to the membrane, where it is either secreted or recycled to the recycling endosomes (Fig. 7). Our data suggest that hypoxia shifts the direction of TNF-α from the membrane toward the lysosome, where its degradation is increased. As increased colocalization of TNF-α and Rab7 did not occur in these conditions, we speculate that TNF-α is directly transported via Rab5-carrying endosomes to the lysosomes, thus bypassing the need for late endosomes, as has been observed before (1). Furthermore, to the best of our knowledge, this is the first time that secretion of TNF-α is localized to secretory lysosomes, which are specialized vesicles that are specifically used by immune cells (14, 18).

In addition to intracellular and membranal events, extracellular mechanisms could also lead to reduced TNF-α levels in hypoxic supernatants. Enzymes such as cathepsins, elastase, and stress-induced proteases were shown to degrade TNF-α in supernatants and produce inactive fragments of the cytokine (3, 22, 31). However, we ruled out the possibility that hypoxia enhanced extracellular degradation of TNF-α, since we detected only the mature form of TNF-α (17 kDa) and no additional degradation products in hypoxic supernatants. An additional explanation for the reduced amounts of TNF-α and its biological activity in hypoxic supernatants could be its enhanced binding to the soluble TNF receptors I and II (sTNF-R1, sTNF-RII). These receptors can bind and neutralize secreted or membranal TNF-α (44), as well as induce reverse signaling in monocytes expressing membranal TNF-α that causes LPS resistance and downregulation of TNF-α secretion (11). Hypoxia and trauma were both shown to enhance the release of the soluble TNF receptors, mainly sTNFR-II (8, 15, 36, 41), although LPS-induced TNFα levels were not always reduced. Here we show that despite the failure of hypoxia to increase the secreted amounts of the two soluble receptors, the binding of TNF-α to sTNFR-II, but not to sTNFR-I, was enhanced, suggesting that this receptor binds and sequesters the secreted cytokine. Since the expression and secretion of sTNFR-II remain unaffected by hypoxia, we speculate that hypoxia causes a change in one or more posttranslational modifications, in either the receptor or in TNF-α itself, which may change the binding affinity of the ligand to its receptor.

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REFERENCES


