Nitrergic oxide is synthesized by nitric-oxide synthase from arginine, a common substrate of arginase. Rat peritoneal macrophages were cultured in the presence of bacterial lipopolysaccharide (LPS), and expression of the inducible isofrom of nitric-oxide synthase (iNOS) and liver-type arginase (arginase I) was analyzed. mRNAs for iNOS and arginase I were induced by LPS in a dose-dependent manner. iNOS mRNA appeared 2 h after LPS treatment and increased to a near maximum at 8–12 h. On the other hand, arginase I mRNA that was undetectable prior to the treatment began to increase 4–6 h with a lag time and reached a maximum at 12 h. Immunoblot analysis showed that iNOS and arginase I proteins were also induced. mRNA for arginase II, an arginase isozyme, was not detected in the LPS-activated peritoneal cells. mRNA for CCAAT/enhancer-binding protein β (C/EBPβ), a transactivator of the arginase I gene, was also induced, and the induction was more rapid than that of arginase I mRNA. Changes in iNOS and arginase I mRNAs were also examined in LPS-injected rats in vivo. iNOS mRNA increased rapidly in the lung and spleen, reached a maximum 2–6 h after the LPS treatment, and decreased thereafter. Arginase I mRNA was induced markedly and more slowly in both tissues, reaching a maximum in 12 h. Thus, arginase I appears to have an important role in down-regulating nitric oxide synthesis in murine macrophages by decreasing the availability of arginine, and the induction of arginase I is mediated by C/EBPβ.

Nitrergic oxide (NO)1 is a major molecule regulating blood vessel dilatation and immune response and functions as a neurotransmitter in the brain and peripheral nervous system (see Refs. 1–3 for reviews). NO is synthesized from arginine by nitric-oxide synthase (NOS), generating citrulline. Cellular NO production is absolutely dependent on the availability of arginine, where arginine is synthesized from citrulline and released into the blood circulation (see Ref. 4 for a review). In other tissues and cell types, arginine can be generated from citrulline, which is produced as a coproduct of the NOS reaction, forming a cycle that is composed of NOS, argininosuccinate synthetase, and argininosuccinate lyase that is termed the “citrulline-NO cycle” (5–10). The inducible isoform of NOS (iNOS) and argininosuccinate synthetase are coinduced in activated murine macrophage-like RAW 264.7 cells (8), in cultured vascular smooth muscle cells (9), and in vivo (10, 11). Argininosuccinate lyase is also induced in vivo (10, 11).

On the other hand, arginase is utilized for both the arginase and NOS reactions. Thus, these two enzymes compete for arginine. At least two isofroms of arginase are present. Liver-type arginase (arginase I) is expressed almost exclusively in the liver and catalyzes the final step of urea synthesis. Arginase I is induced by dietary protein (14) and hormones (15). Arginase I consists of three identical subunits of about 35,000 Da. cDNA clones were isolated from rat (16, 17) and human (16, 17) liver. The rat (20) and human (21) genes are 11.5–12 kb long and consist of 8 exons. Promoter and enhancer regions of the rat gene were characterized (22–24). In addition to arginase I, an isozyme (arginase II) is present in extrhepatic tissues, including kidney, small intestine, and lactating mammary gland (25–27). The coinduction of NOS and arginase II activities in RAW 264.7 cells activated by lipopolysaccharide (LPS) was reported (28). We isolated cDNA for arginase II and showed that it is 59% identical with arginase I on the amino acid level (29). We also found that the enzyme is mitochondrial and that its mRNA is induced by LPS, dexamethasone, and cyclic adenosine monophosphate and is reduced by interferon-γ (29). To better understand the role of arginase isoforms in NO synthesis, we examined expression of the isoforms and iNOS in cultured rat peritoneal cells and in LPS-treated rats using RNA blots, immunoblots, and immunocytochemical analyses. We report here that iNOS and arginase I mRNAs and proteins are coinduced by LPS in cultured rat peritoneal macrophages and in the lung and spleen in vivo. The induction of mRNA for CCAAT/enhancer-binding protein β (C/EBPβ), a transactivator of the arginase I gene, is also described.

EXPERIMENTAL PROCEDURES

Materials—A monoclonal antibody against mouse iNOS was obtained from Transduction Laboratories (Lexington, KY). Antiserum

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against human arginase I was raised in a rabbit. Purified recombinant human arginase I (30) was provided by M. Ikemoto of Kyoto University, Kyoto, Japan. A monoclonal antibody RM4 against rat macrophages will be described elsewhere (41).

Preparation and Culture of Rat Peritoneal Cells—Specific pathogen-free male Wistar rats (7 weeks of age) were given 5 ml of 10% polypeptide (Difco) intraperitoneally, and peritoneal cells were harvested 3 days after the injection (31). The cells were seeded in 10-cm culture dishes at 5 × 10^5 cells/dish in RPMI 1640 medium with 10% heat-inactivated fetal calf serum. The cells were cultured in the absence or presence of LPS at 37°C under 5% CO₂ in air.

Animals and LPS Treatment—Male Wistar rats (5–6 weeks of age) were given Escherichia coli LPS (serotype 0127:B8, Sigma) intraperitoneally at 1.0 mg/kg of body weight and killed at the indicated times and total RNAs (0.5 μg) were subjected to blot analysis. The results in A were quantified using a bioimaging analyzer and are represented by mean ± S.D. Maximal values are set at 100.

RNA Blot Analysis—Total RNA from rat tissues and packed peritoneal cells was prepared by the guanidium thiocyanate-phenol-chloroform extraction procedure (32). After electrophoresis in formaldehyde-containing agarose gels, RNAs were transferred to nylon membranes. Hybridization was performed using as probes digoxigenin-labeled antisense RNAs for rat iNOS (10), rat arginase I, mouse C/EBPα, or rat arginase II. The antisense RNAs were synthesized using as templates pcDNAII-riNOS (10), pcDNAII-rAI-1, pcDNAII-C/EBPα, and pGEM-rAlI-1 (29), respectively. To obtain the plasmid pcDNAII-rAI-1, the approximately 850-bp EcoRI-EcoRV fragment of pARGr-2 (17) was inserted into the EcoRI and EcoRV sites of the plasmid pcDNAII (Invitrogen, San Diego, CA). To obtain the plasmid pcDNAII-C/EBPα, the approximately 1.6-kb BstXI fragment of pEF-C/EBPα (33) was inserted into the BstXI site of the pcDNAII. Chemiluminescence signals derived from hybridized probes were detected on x-ray films using a DIG luminescence detection kit (Boehringer Mannheim) and quantified using the MacBas bioimage analyzer (Fuji Photo Film Co., Tokyo, Japan).

Immunoblot Analysis—Rat tissues were excised and homogenized in 9 volumes of 20 mM potassium HEPES buffer, pH 7.4, containing 1 mM dithiothreitol, 50 μM antipain, 50 μM leupeptin, 50 μM chymostatin, and 50 μM pepstatin. Packed rat peritoneal cells were homogenized in 9 volumes of the same buffer containing 0.5% Triton X-100. The homogenates were centrifuged at 25,000 × g for 30 min at 4°C and the supernatants were used as tissue or cell extracts. The extracts were subjected to SDS-polyacrylamide gel electrophoresis and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham Corp.) according to the manufacturer’s protocol. Protein was determined with protein assay reagent (Bio-Rad) using bovine serum albumin as a standard.

Immunocytochemical Staining—Cultured peritoneal cells were prepared for immunocytochemical staining by cytocentrifuge. Briefly, 5 × 10^6 cells were suspended in 100 μl of RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, and then the cell suspension was applied on cytoospin slides by centrifugation at 600 rpm for 5 min (Cytospin 2, Shandon, Astmoor, United Kingdom). Immunocytochemical staining was done as described (10).

RESULTS

Induction by LPS of iNOS and Arginase I mRNAs in Rat Peritoneal Cells—Rat peritoneal cells were cultured in the presence of LPS, and expression of iNOS and arginase I mRNAs was studied. Fig. 1 shows the effects of LPS concentration on iNOS and arginase I mRNAs. iNOS mRNA of about 4.5 kb, which was not detectable in the absence of LPS, was evident at 1 ng/ml and gradually increased with increasing concentrations of LPS up to 10 μg/ml. mRNA for arginase I (liver-type arginase) of about 1.7 kb was also induced by LPS. However, dose dependence differed from that of iNOS mRNA. Arginase I mRNA was barely detectable with 1 ng/ml of LPS; it
was detected with 10 ng/ml and the level increased with up to 10 μg/ml. The maximal mRNA concentration seen with 10 μg/ml of LPS was comparable to that in the liver.

Fig. 2 shows the time course of induction of iNOS and arginase I mRNAs in the peritoneal cells treated with 10 μg/ml of LPS. iNOS mRNA was detected at 2 h, increased with time, reached a near maximum at 8 h and a maximum at 12 h, and remained at the near maximal level at 24 h. On the other hand, arginase I mRNA began to increase after 4 h with a lag time and reached a maximum at 12–24 h. Thus, iNOS and arginase I mRNAs are coinduced by LPS in rat peritoneal cells, but arginase I mRNA is induced more slowly than iNOS mRNA. Arginase II mRNA, which is 57% identical with arginase I mRNA, was not detected in LPS-treated peritoneal cells under conditions where the mRNA of 1.8 kb was clearly detected in the rat intestine (data not shown).

**Induction of C/EBPβ mRNA in Peritoneal Cells**—We reported that C/EBP family members bind to both the promoter and enhancer regions of the rat arginase I gene (22, 23) and that the promoter is activated by C/EBPβ and other members (24). We then asked whether C/EBPβ mRNA would also be induced by LPS in rat peritoneal cells. mRNA was present at a very low level prior to the LPS treatment; it increased with time, reached a maximum at 12 h, and then decreased (Fig. 2, A, c, and B, c). Thus, induction of C/EBPβ mRNA was more rapid than that of arginase I mRNA.

**Induction of iNOS and Arginase I Proteins in LPS-treated Peritoneal Cells**—Fig. 3 shows the time course of induction of iNOS and arginase I proteins in cultured peritoneal cells after exposure to LPS. iNOS protein of about 150 kDa was induced 8 h after the treatment; it increased at 24 h, then decreased. On the other hand, two polypeptides of about 35 and 38 kDa immunoreacted with the arginase I antibody, comigrated with the two forms of arginase I in the liver, and were observed 8 h after the treatment, then increased to 48 h. These two polypeptides of arginase I apparently arose by alternative translation initiation from the two methionine residues located 30 base pairs apart (17, 20, 34). Concentration of arginase I protein in the LPS-treated peritoneal cells was comparable to that in the liver; these findings were in accord with the observation that mRNA concentrations in the activated peritoneal cells and in the liver were comparable (see above).

**Immunocytochemical Detection of iNOS and Arginase I in Peritoneal Cells**—To identify cells positive for iNOS and arginase I in peritoneal cells, immunocytochemical analysis was performed (Fig. 4). iNOS immunoreactivity was absent prior to LPS treatment. However, most cells became strongly positive for iNOS immunoreactivity after the treatment. Arginase I was negative prior to the LPS treatment, but most cells became positive after the treatment. Distributions of the iNOS-positive cells, the arginase I-positive cells, and macrophages positive to the macrophage-specific antibody RM4 were similar. However, cellular staining patterns of iNOS and arginase I differed from that with antibody RM4; iNOS and arginase I are cytosolic proteins, whereas the antibody RM4 is specific to lysosomes in macrophages. Therefore, iNOS and arginase I are coinduced in practically all peritoneal macrophages.

**Induction of mRNAs and Proteins for iNOS and Arginase I in the Lung and Spleen of LPS-treated Rats**—We then examined expression of iNOS and arginase I in vivo. We previously reported that iNOS mRNA was induced in various tissues of rats injected intraperitoneally with LPS (10); the induction was the strongest in the lung and spleen. mRNAs for arginase I as well as for iNOS in the lung were measured at various times after LPS treatment (Fig. 5). iNOS mRNA in the lung increased to a near maximum 2 h after the treatment, reached a maximum at 6 h, decreased thereafter, and returned to a hardly detectable level at 24 h. Arginase I mRNA was also induced. mRNA was present at a very low level before treatment, began to increase after 2 h with a lag time, reached a maximum at 12 h on the average, and then decreased slowly. Concentration of the mRNA at 12 h was over 10-fold lower than that in the liver (data not shown).

iNOS mRNA in the spleen showed an increase similar to that in the lung, but the level decreased more slowly than seen in the lung (10). Arginase I mRNA was also induced in the spleen, but less markedly than in the lung, and the time course was similar to that in the lung and slower than that of iNOS mRNA in this organ (data not shown).

We reported that iNOS protein was markedly induced in the lung and spleen of LPS-treated rats (10). The induction of arginase I protein in the lung was also examined using immunoblot analysis. A polypeptide that comigrated with the larger form of arginase I was induced 24 h after LPS-treatment (data not shown). Concentration of arginase I in the
lung of the LPS-treated rat was over 10-fold lower than that in the liver.

DISCUSSION

NO synthesis is regulated by depending on the availability of arginine, substrate of the NOS reaction, as well as by NOS activity and other factors. Arginine can be obtained via the blood circulation or by endogenous synthesis from citrulline. Arginine transport into cultured macrophages increases in response to LPS and to interferon-γ (35, 36). The cat-2 gene of the arginine transporter in cultured vascular smooth muscle cells is stimulated by interleukin-1β and by tumor necrosis factor-2 (37). Furthermore, argininosuccinate synthetase and arginino- succinate lyase, which together synthesize arginine from citrulline, are induced in stimulated murine macrophages (8, 10) and aortic smooth muscle cells (9), and intracellular arginine synthesis is enhanced.

Activity of arginase that degrades arginine is also induced in activated macrophages (38, 39). Arginase induced in activated RAW 264.7 cells, a mouse macrophage-like cell line, was shown to be arginase II (28, 29). On the other hand, in the present work, we noted that arginase I, not arginase II, is induced by LPS in primary cultured rat peritoneal macrophages and in the lung and spleen in vivo. This was unexpected because arginase I has been thought to be expressed almost exclusively in the liver. Differential induction of the two arginase isoforms in the mouse macrophage-like cell line and in primary cultured rat macrophages may be due to differences in animal species or to differences between the established cell line and the primary cultured cells. Arginase I mRNA was induced more slowly than iNOS mRNA. The induced arginase I probably decreases arginine availability for the NOS reaction in activated macrophages and may down-regulate the overproduction of NO. All of these results suggest that there is a complex regulation of genes encoding enzymes and transporter proteins involved in arginine metabolism that together control NO production in cells.

Arginase I mRNA was induced slowly in cultured peritoneal macrophages and also in vivo with an apparent lag time of a few hours. Therefore, the induction of arginase I appears to be mediated by a transcription factor(s) that is synthesized de novo in response to LPS stimulation. C/EBP family members bind to the promoter and enhancer regions of the arginase I gene and activate the promoter (Refs. 22–24; see Ref. 40 for a review), C/EBPβ being the most potent. The present study also shows that C/EBPβ mRNA is induced in the LPS-stimulated peritoneal macrophages. The induction of C/EBPβ mRNA is more rapid than that of arginase I mRNA. Thus, the induction of arginase I appears to be mediated, at least in part, by the induction of C/EBPβ.

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