Nitric oxide (NO) is synthesized from arginine by nitric oxide synthase (NOS), and citrulline which is generated can be recycled to arginine by argininosuccinate synthetase (AS) and argininosuccinate lyase (AL). Rats were injected with bacterial lipopolysaccharide (LPS), and expression of the inducible isoform of NOS (iNOS), AS, and AL was analyzed. In RNA blot analysis, iNOS mRNA was undetectable before the LPS treatment but was induced by LPS in the lung, heart, liver, and spleen, and less strongly in the skeletal muscle and testis. AS mRNA was induced in the lung and spleen, and AL mRNA was weakly induced in these tissues. AS and AL mRNAs were abundant in the control liver and remained unchanged after the treatment. Kinetic studies showed that iNOS mRNA increased rapidly in both spleen and lung, reached a maximum 2–5 h after the treatment, and decreased thereafter. On the other hand, AS mRNA increased more slowly and reached a maximum in 6–12 h (by about 10-fold in the spleen and 2-fold in the lung). AL mRNA in the spleen and lung increased slowly and remained high up to 24 h. In immunoblot analysis, increase of iNOS protein was evident in the lung, liver, and spleen, and there was an increase of AS protein in the lung and spleen. In immunohistochemical analysis, macrophages in the spleen that were negative for iNOS and AS before LPS treatment were strongly positive for both iNOS and AS after this treatment. As iNOS, AS, and AL were coinduced in rat tissues and cells, citrulline-arginine recycling seems to be important in NO synthesis under the conditions of stimulation.

Nitric oxide (NO) is a major messenger molecule regulating blood vessel dilatation and immune function 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 as another product. Cellular NO production is absolutely dependent on availability of arginine. This amino acid can be obtained from exogenous sources via the blood circulation, from intracellular protein degradation, or by endogenous synthesis of arginine. Major sites of arginine synthesis in ureotelic animals are the liver, where arginine generated in the urea cycle (ornithine cycle) is rapidly converted to urea and ornithine by arginase, and the kidney, where arginine is synthesized from citrulline and released into the blood circulation (see Ref. 4 for a review). However, other tissues and cell types also contain low levels of argininosuccinate synthetase (AS) and argininosuccinate lyase (AL), which together synthesize arginine from citrulline (5–8). Therefore, arginine can be generated from citrulline which is produced as a co-product of the NOS reaction, forming a cycle which could be termed the “citrulline-NO cycle” (9) or “arginine-citrulline cycle” (10). Vascular endothelial cells can convert citrulline to arginine (11), and this conversion is increased when cells are stimulated to produce NO (12). Cytokine-activated macrophages, which produce a large amount of NO, have an increased capacity to produce arginine from citrulline (13). Furthermore, the inducible isoform of NOS (iNOS) and AS are coinduced in a murine macrophage cell line (9) and cultured vascular smooth muscle cells (10). AS (14–16) and AL (17–20) have been purified and characterized. cDNAs and genomic clones for AS (21–23) and AL (24–27) were isolated, and the promoters of the AS gene (28) and the AL gene (29) were characterized. To better understand the role of these citrulline-NO cycle enzymes in NO synthesis in vivo, we examined expression of the enzymes in lipopolysaccharide (LPS)-treated rats using RNA blots, immunoblots and immunohistochemical analyses. We report here that iNOS, AS, and AL are coinduced by LPS in the spleen and lung of rats. Immunohistochemical analysis revealed induction of both iNOS and AS in macrophages of the spleen after LPS treatment.

**EXPERIMENTAL PROCEDURES**

Materials—A monoclonal antibody against mouse iNOS was obtained from Transduction Laboratories, Lexington, KY. Antisera against rat AS and AL were as reported elsewhere (8). Preparation of a monoclonal antibody RM4 against rat macrophages will be published elsewhere. Animals and LPS Treatment—Specific pathogen-free male Wistar rats (5–6 weeks of age) were injected intraperitoneally with Escherichia coli LPS (serotype 0127:68, Sigma) at 1.0 mg/kg body weight, and then killed following anesthetization with ether.

RNA Blot Analysis—Total RNA from rat tissues was prepared by the guanidium thiocyanate-phenol-chloroform extraction procedure (30).
After electrophoresis in formaldehyde-containing agarose gels, RNAs were transferred to nylon membranes (Boehringer Mannheim). Hybridization was performed using as probes digoxigenin-labeled rat iNOS antisense RNA (Ref. 31; nucleotides 2344–3026), rat AS antisense RNA (8), or rat AL antisense RNA (8). The iNOS antisense RNA was synthesized using iNOS cDNA that was cloned using reverse transcription-polymerase chain reaction and subcloned into pCDNAI, as templates and the DIG-RNA labeling kit (Boehringer Mannheim). Chemiluminescence signals derived from hybridized probes were detected on x-ray films using DIG luminescence detection kits (Boehringer).

Immunohistochemical Staining—Rat tissues were excised and homogenized in nine 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. The homogenates were centrifuged at 25,000 × g for 30 min at 4 °C, and the supernatants were used as tissue extracts. The tissue extracts were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham) according to the protocol supplied by Amersham. Chemiluminescence signals on x-ray films were quantified by densitometry.

Immunoblot Analysis—Rat tissues were excised and homogenized in nine 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. The homogenates were centrifuged at 25,000 × g for 30 min at 4 °C, and the supernatants were used as tissue extracts. The tissue extracts were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham) according to the protocol supplied by Amersham. Chemiluminescence signals on x-ray films were quantified by densitometry.

Induction of iNOS, AS, and AL mRNAs in LPS-treated Rat Tissues—Rats were injected intraperitoneally with bacterial LPS, and iNOS mRNA in various tissues was measured by RNA blot analysis 6 h after the treatment. iNOS mRNA was not detected in any tissue before the treatment (Fig. 1A). However, the mRNA of about 4.5 kb was induced most strongly in the lung and spleen, followed by the heart and liver, and less strongly in skeletal muscle and testis, but apparently not in the brain and kidney.

AS and AL mRNAs were measured in the lung, spleen, and liver of the same rats in which iNOS mRNA was markedly induced. AS mRNA of about 1.5 kb was detected in the lung before the LPS treatment and increased by the treatment (Fig. 1B). AS mRNA was present at a lower level in the spleen than in the lung before the treatment, and increased markedly by the treatment. In the liver where AS is involved in urea synthesis, AS mRNA was much more abundant and was not induced by LPS. AL mRNA of about 2.0 kb was also detected in the lung and spleen before the LPS treatment, and increased weakly in these tissues after the treatment (Fig. 1C). It was much more abundant in the liver where AL is also involved in urea synthesis and remained unchanged by the treatment.

Kinetics of Induction of iNOS, AS, and AL mRNAs in the Spleen—mRNAs for iNOS, AS, and AL in the spleen were examined at various times after the LPS treatment (Fig. 2). iNOS mRNA 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. On the other hand, AS mRNA started to increase after 2 h with a time lag, reached maximum at 6 h on the average (about 10-fold increase), increased further or decreased at 12 h depending on the animal, and returned to control levels at 24 h. AL mRNA increased with kinetics similar to that for AS mRNA, although the increase was less marked (about 2.5-fold)
Fig. 3. Time course of induction of iNOS (a), AS (b), and AL (c) mRNAs in the lung. Total RNA was isolated from the rat lung and RNA blot analysis was performed as described in the legend for Fig. 2.

levels were retained for 24 h. These results show that iNOS, AS, and AL are coinduced by LPS in the rat spleen. iNOS mRNA was induced rapidly and strongly, whereas AS and AL mRNAs were induced somewhat more slowly and AL mRNA was weakly induced. Induction varied from one animal to another, but the three mRNAs behaved similarly in each animal. For example, animal 3 at 6 h (6 h lanes) and animal 1 at 12 h (12 h lanes) were relatively high for all mRNAs.

Kinetics of Induction of iNOS, AS, and AL mRNAs in the Lung—Time course of induction of iNOS, AS, and AL mRNAs in the lung after the LPS treatment is shown in Fig. 3. iNOS mRNA reached a maximum at 2 h and remained at near-maximal levels at 6 h, then decreased slowly, and was still detectable at 24 h. Thus, the mRNA in the lung increased similarly as that in the spleen, and decreased more slowly than that in the spleen. AS mRNA decreased somewhat 2 h after the treatment, increased at 6–12 h by about 2-fold on the average, and then decreased. The induction of AS mRNA was again slower than that of iNOS mRNA and was much less marked than that of AS mRNA in the spleen. AL mRNA in the lung increased gradually up to 12 h by about 2-fold and remained at near-maximal levels at 24 h. The profile of AL mRNA induction in the lung resembled that in the spleen.

Induction of iNOS and AS Proteins by LPS—Induction of iNOS and AS proteins in LPS-treated rat tissues was examined using immunoblot analysis. iNOS protein of about 150 kDa that was undetectable before the LPS treatment, was induced 12 h after the treatment in the lung, liver, and spleen, but apparently not in the kidney (Fig. 4A). These results parallel findings for iNOS mRNA. On the other hand, AS protein of 46 kDa was detected in the lung before the LPS treatment, and increased 12 h after the treatment (Fig. 4B). It was present in a lower amount in the spleen than in the lung before the LPS treatment and increased markedly by the treatment. Again, these results parallel those of AS mRNA.

Immunohistochemical Detection of Spleen Macrophages Positive for iNOS and AS—To identify cells positive for iNOS and AS, immunohistochemical analysis of the spleen was performed (Fig. 5). iNOS immunoreactivity was nil in the spleen prior to LPS treatment. However, a strong iNOS immunoreactivity was found in macrophages in the red pulp of the spleen 12 h after the LPS treatment. AS immunoreactivity was absent before the LPS treatment, and after the treatment, macrophages in the red pulp became strongly positive for AS immunoreactivity. Distribution and staining patterns of iNOS-positive macrophages and AS-positive macrophages were similar. Distributions of these iNOS- and AS-positive cells were similar to macrophages positive with a macrophage-specific antibody RM4. However, staining patterns of iNOS- and AS-positive cells differed from that of RM4-positive cells, perhaps because iNOS and AS are cytosolic enzymes, whereas the antibody RM4 preferentially stains lysosomes in macrophages. These results suggest that iNOS and AS are colocalized in all macrophages in the spleen. No change was found in the distribution of RM4-positive cells before and after LPS treatment. These results suggest that iNOS and AS are induced in macrophages that are already present before the treatment. On the other hand, AL immunoreactivity was not found before and after the LPS treatment. This apparent lack of AL immunoreactivity in macrophages may be due to a small amount of the AL protein and a low induction of the protein (about 2.5-fold induction at the mRNA level).

DISCUSSION

Cellular NO production is determined by NOS activity, intracellular arginine concentration, and other factors. Induction of iNOS in response to various stimuli has been reported for a variety of cell types in vitro (33–35) and also in rat tissues in vivo (36–38). Arginine can be supplied via the blood circulation or regenerated from citrulline by the citrulline-NO cycle that is composed of iNOS, AS, and AL. The coinduction of iNOS and AS was noted in a murine macrophage cell line (9) and a murine aortic smooth muscle cell line (10), thereby suggesting...
the importance of the citrulline-NO cycle, at least in these cell types. The present study shows for the first time that iNOS, AS, and AL are coinduced by LPS in vivo. Coinduction of iNOS and AS was evident in the rat spleen and lung at both mRNA and protein levels. AL mRNA was induced weakly in the spleen and lung. iNOS mRNA was highly induced in the liver, but AS and AL mRNAs were abundant in this tissue prior to LPS treatment and did not increase further. This can be explained by the roles of AS and AL in urea synthesis in the liver. iNOS mRNA was induced also in the heart, muscle and testis, but apparently not in the brain and kidney. Ohshima et al. (36) reported that iNOS protein was induced in the liver, lung, and spleen of the rat by administration of Propionibacterium acnes and LPS. Hom et al. (37) reported that iNOS mRNA and protein were induced by LPS in many tissues of the rat, including the brain and kidney. Sato et al. (38) reported that iNOS protein was induced in the lung, liver, spleen, and peritoneal macrophages of the LPS-treated rats. The minor discrepancies in these studies may be due to differences in rat strain, amount of LPS, and route of LPS administration.

Kinetic studies showed that iNOS mRNA is induced very rapidly with little time lag both in the spleen and lung, whereas AS mRNA is induced more slowly with a time lag of about 2 h in these tissues. Induction of AL mRNA in these tissues is also slow, albeit less marked. A rapid induction of NOS activity, followed by a delayed induction of AS activity in the rat aortic smooth muscle cells treated with LPS and interferon-γ, was also reported (10). This means that the expression of the iNOS gene and those of the AS and AL genes are partly coordinated and partly dissociated, possibly because of different roles of these enzymes; iNOS is involved only in NO synthesis, whereas AS and AL are responsible to synthesis of arginine, a precursor for synthesis of proteins, polyamines, and creatine phosphate in addition to NO. Studies on regulatory elements of the iNOS, AS, and AL genes and trans-acting factors will need to be done.

An immunohistochemical study of iNOS in LPS-treated rats (39) showed that iNOS immunoreactivity was strongly detected in macrophages in the heart, lung, liver, and kidney 6–9 h later. Another immunohistochemical study (38) revealed that inflammatory cells in many tissues, hepatocytes, and endothelial cells of the aorta of rats were positive for iNOS around 6 h after LPS injection, and that macrophages were positive in the liver and spleen after 12 h. The present immunohistochemical study shows that the iNOS and AS are colocalized in the spleen macrophages of LPS-treated rats. All these results suggest strongly that the citrulline-NO cycle functions in vivo in macrophages of the spleen and perhaps in other tissues and cell types.
types. Further immunohistochemical and in situ hybridization analyses are under way.

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