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EFFECT OF LIPOPOLYSACCHARIDE TREATMENT *IN VIVO* ON TISSUE EXPRESSION OF ARGININOSUCCINATE SYNTHETASE AND ARGININOSUCCINATE LYASE mRNAs: RELATIONSHIP TO NITRIC OXIDE SYNTHASE

Yoshiyuki Hattori[1,*], Shin-Ichi Shimoda[1] and Steven S. Gross[2]

[1]Department of Endocrinology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan

[2]Department of Pharmacology, Cornell University Medical College, New York, NY 10021

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SUMMARY: Since arginine is the only physiological substrate for the NO synthase reaction, regulation of arginine availability could determine the cellular rate of NO production. We investigated whether lipopolysaccharide (LPS) treatment *in vivo* would alter tissue expression of mRNAs for argininosuccinate synthetase (AS) and argininosuccinate lyase (AL), the net action of which is to convert citrulline to arginine. Concomitant with the induction of NO synthase mRNA, injection of LPS into the rats elicited an increase in AS and AL mRNA levels in the tissues. In contrast with modest increases in the abundance of AS and AL mRNA in lung and heart, a marked increase in levels of AS and AL mRNA in the kidney occurred. The liver, whether or not treated with LPS, contained high levels of mRNA for AS and AL which are components of the urea cycle. Findings suggest that an increase in the renal capacity to convert citrulline to arginine could play a key role in NO formation *in vivo* when arginine becomes limiting.

As for the possible organ sites of arginine biosynthesis, it has long been known that the kidney is capable of converting citrulline to arginine (1,2). The renal release of arginine into the circulation matches precisely the renal uptake of citrulline from the blood (3). Biosynthesis of arginine in the kidney is catalyzed by argininosuccinate synthetase (AS) and argininosuccinate lyase (AL) (1,2). While both enzymes are present in the liver as components of the urea cycle, but high hepatic levels of arginase limit the release of arginine into the circulation (1,2). Thus, while these enzymes are part of a catabolic pathway in the liver, they comprise an anabolic pathway in the kidney.

With the discovery of arginine-derived nitric oxide (NO) as a key cellular signaling molecule, the possibility arises that the function of AS and AL is to convert citrulline, a co-product of the NO synthase (NOS) reaction, back to arginine for the continued production of NO. Indeed, two NO-

* To whom correspondence should be addressed: Department of Endocrinology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, Japan. FAX: 0282-86-4632.

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producing types of cells, the bovine aortic endothelial cells and the rat peritoneal macrophages, reportedly synthesize arginine from citrulline and this conversion is increased when the cells are stimulated to produce NO (4,5). More recently, we and other investigators demonstrated that AS mRNA and activity are induced by immunostimulants in cultured vascular smooth muscle and in a murine macrophage cell line (6,7). In both cell types, arginine is regenerated from citrulline to form an "arginine-citrulline cycle" or a "citrulline-NO cycle" (6,7).

The induction of NOS or the overproduction of NO are implicated in the genesis of septic and cytokine-induced circulatory shock *in vivo* (8,9). Little is known, however, about alterations in the availability of arginine under such conditions. In the present study, we measured the abundance of AS and AL mRNA in lung, heart, liver, and kidney, using a model of LPS injection in rats.

MATERIALS AND METHODS

Animal treatment and extraction of RNA

Male Wistar rats (250-300 g) were injected intravenously with either LPS (*E.coli* type, Serotype 0111:B4, 10 mg/kg: Sigma Chemical Co., St. Louis. MO, USA) or saline (1ml/kg) in the endotoxin and control groups, respectively. The animals were sacrificed three hours later by exsanguination, and various organs were removed, immediately frozen in liquid nitrogen, and stored at -70°C until RNA extraction. Total RNA was extracted from lung, heart, liver and kidney by the guanidinium isothiocyanate/acid phenol method (10).

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed by standard methods as previously described (11). The first strand of cDNA was synthesized from 1mg of RNA by use of random primers and M-MLV reverse transcriptase (Promega, Madison, WI, USA), followed by PCR amplification with synthetic gene-specific primers for rat AS (6), AL (6), and murine-inducible NOS (iNOS) (12). Primers used were: AS forward 21-mer, 5'-TGGAGGATGCCCGAGTTTTAC-3'; AS reverse 21-mer, 5'-CTTTCCTTCCACCCGTTCCTG-3'; AL forward 21-mer, 5'-GCGGAGTGTGAAGTCCTCTTC-3'; AL reverse 21-mer, 5'-TCCAGTGGCTACTTGGAGGAC-3'; iNOS forward 21-mer, 5'-CTGCAGGTCTTTGACGCTCGG-3'; iNOS reverse 21-mer, 5'-GTGGAACACAGGGGTGATGCT-3'. PCR amplification was performed with a DNA PCR kit (Perkin Elmer, Norwalk, CT, USA) according to the following schedule: denaturation, annealing, and elongation at 95, 55, and 72°C for 30s, 30s, and 1 min, respectively, for 30 cycles. Parallel amplification of rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was performed for reference using primers as described (13). Possible contamination of any PCR component was excluded by carrying out a PCR reaction with these components in the absence of RT product in each set of experiments (negative control). To verify that amplification did not proceed from residual genomic DNA, a control PCR reaction was carried out for each RNA, using RNA as the template.

Analysis of PCR products

PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and were visualized by UV-induced fluorescence. The intensity of the PCR bands on the negative film of the gel photographs was quantified by use of a video densitometer linked to a computer analysis system (ACI, Kanagawa, Japan).

RESULTS

The PCR products resulting from use of AS- and AL- specific primers showed clear bands at the predicted sizes of 612 and 600 bp, respectively (Fig.1). These bands were absent from the PCR-amplified products when RNA was used as a template or from those lacking a cDNA template. This finding indicates that the 612- and 600-bp bands originated from mRNA, not from genomic DNA or other contaminant. To confirm the identity of the PCR products as AS and AL, they were cut with Echo RI and each was found to give two fragments of the expected sizes, based on reported sequences for rat AS and AL (Fig.1).

The iNOS mRNA was markedly increased in the lung, heart, liver and kidney from LPS-treated rats, but was absent from all tissues studied in control rats (Fig.2A). The AS signal was absent or negligible in the control lung and heart, whereas a large signal was detected in the control liver and kidney. After LPS, the AS signal remained elevated in the liver and was further increased in the kidney. Although the AL signal was very low in the control lung and heart, we observed a large signal in the control liver and a moderate signal in the control kidney. After LPS, the AL signal remained elevated in the liver and was markedly increased in the kidney. A modest induction of AS and AL signals was observed in both lung and heart after LPS. For quantification, we normalized the AS and AL signals relative to the corresponding GAPDH signal from the same RNA; they were expressed as AS/GAPDH and AL/GAPDH, respectively. The AS/GAPDH ratios and the AL/GAPDH ratios for lung, heart, liver and kidney are shown in Fig.2B.

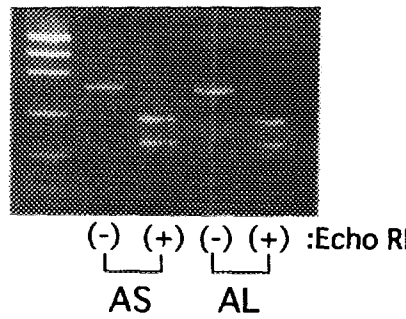

Fig.1. PCR products for AS and AL derived from AS and AL mRNA in rat kidney. The PCR products were cut with Echo RI. DNA size markers in the left lanes correspond to 2000, 1200, 800, 400, 200, and 100 bp.

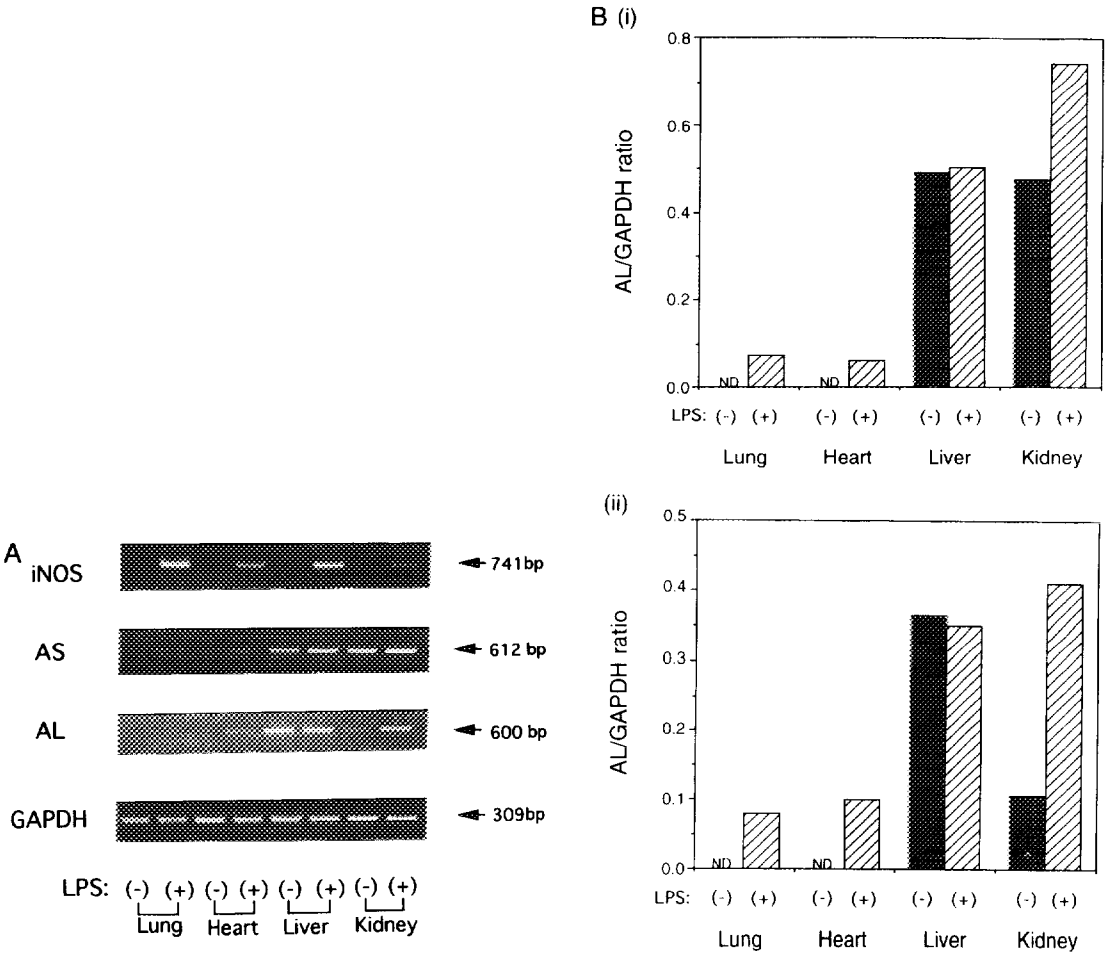

Fig.2. Effects of LPS treatment *in vivo* on AS, AL and iNOS mRNA expression in lung, heart, liver and kidney. Total RNA was prepared from tissues of rats 3h after the intravenous administration of saline (control: LPS(-)) or of LPS (10 mg/kg: LPS(+)), and assayed by RT-PCR using gene-specific primers. Results using primers specific for GAPDH are shown for comparison. (A) Representative photographs are shown; arrows indicate the predicted size of PCR products. (B) Bar graph showing the relative amounts of AS (i) and AL (ii) mRNA quantified by densitometry and expressed as AS/GAPDH or AL/GAPDH ratio (optical density ratio of AS or AL to GAPDH). Black bars: the control animal, shaded bars: the LPS-treated animal, ND: not detectable.

DISCUSSION

Because the plasma level of arginine limits the synthesis of NO, an enhanced cellular capacity to regenerate arginine from citrulline could be important in the regulation of NO production, especially when iNOS is expressed. LPS treatment *in vivo* induces iNOS in various tissues of the rat when plasma NO_2^-/NO_3^- concentrations and tissue iNOS mRNA and activity are elevated (12,14,15). Concomitant with the induction of iNOS mRNA, as we demonstrated, the injection of LPS elicited

an increase in levels of AS and AL mRNA in the tissues. In contrast with the widespread induction by LPS of iNOS mRNA in rat tissues, the expression and change in the abundance of AS and AL mRNA appeared to be tissue-specific. The present results suggest that endogenous arginine synthesis through the net action of AS and AL normally occur in the liver and, to a lesser extent, in the kidney, and that the capacity for the kidney to regenerate arginine can be up-regulated by administering LPS. The complete urea cycle is expressed only in the liver, where the arginine generated in the urea cycle is rapidly converted to urea and ornithine by arginase, and is, therefore, not released into the circulation (1,2). Indeed, the liver of control and LPS-treated rats contained high levels of mRNA for liver-type arginase (data not shown). Although arginase activity was observed in the kidney, it was much lower than in the liver (1,2). Such activity includes the that of an isozyme distinct from liver arginase (1). Renal arginase activity is segregated from the major site of renal arginine synthesis (1,16). Thus, the kidney appears to be the major site for the regeneration and release of arginine into the circulation. The regeneration of arginine in the kidney could be up-regulated under the conditions in which iNOS is expressed. The release into the circulation or transport of the arginine that is regenerated in the kidney may also be up-regulated under such conditions. In fact, arginine transport in macrophages and other cells increases in response to agents that induce iNOS (17,18). We observed that LPS treatment *in vivo* caused mRNA induction in the rat kidney of iNOS and AS/AL, as well as of the cationic amino acid transporter, system y^+, that allows arginine to enter or leave the cell. The mRNAs for both of the two distinct cationic amino acid transporters which differs in a portion of its sequence, presumably as a result of alternative splicing (19,20), are up-regulated in the kidney after LPS treatment (unpublished data, Hattori), and may contribute to the enhanced release of arginine into the circulation.

It has been shown in cultured cells that iNOS and AS are co-activated by LPS and interferon-γ to form an arginine-citrulline cycle that permits the high output of NO production by the cells (6,7). The present study suggests the importance of this arginine recycling pathway under some conditions *in vivo*. The regulation of arginine availability may be an important target for pharmacological interventions to suppress NO overproduction, i.e. as in septic and cytokine-induced shock. Further study is needed to understand the net action and co-regulation of the factors that alter arginine availability and determine the effect on NO production.

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