Induction of Ca²⁺/calmodulin-dependent NO synthase in various organs of rats by *Propionibacterium acnes* and lipopolysaccharide treatment

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Ca^{2+}/calmodulin-dependent nitric oxide synthase was found to be induced during rat liver necrosis caused by administration of *Propionibacterium acnes* and *E. coli* lipopolysaccharide to rats. Examination of the specific induction of Ca^{2+}/calmodulin-dependent NO synthase showed that the enzyme was induced in the lung, spleen and colon as well as the liver. Northern blot analysis revealed that the induction occurred at the transcriptional level.

Nitric oxide; NO synthase; Calmodulin-dependent NO synthase; Inducible; Rat; Lipopolysaccharide

1. INTRODUCTION

Nitric oxide is known to have many biological functions, such as neural transmission, vasodilatation, cytotoxic actions of macrophages and leukocytes, adaptive relaxation of the stomach, and inhibition of platelet aggregation [1,2]. It is not known, however, how these diverse biological functions are regulated, or how many isozymes of NO synthase (EC 1.14.23) there are.

Isozymes of NO synthase can be classified into two types, constitutive and inducible. All the constitutive isozymes so far reported are Ca^{2+}/calmodulin-dependent [3–6]. An inducible NO synthase has been purified from macrophages and shown to be calmodulin-independent [7,8]. A similar calmodulin-independent NO synthase has also been purified from polymorphonuclear leukocytes [9]. We found that both calmodulin-dependent and -independent NO synthase activities are induced in the liver of rats treated with *Propionibacterium acnes* and lipopolysaccharide (LPS) [10]. Recently, we confirmed the induction of the Ca^{2+}/calmodulin-dependent isozyme by its purification from rats treated as mentioned above (to be published elsewhere) and showed that at least part of the calmodulin-independent isozyme was a calmodulin-bound form of Ca^{2+}/calmodulin-dependent NO synthase (Iida et al., to be published elsewhere). For a better understanding of the biological roles of NO synthase, information is needed on the molecular diversity and the regulation of expression and characteristics of its isozymes. In the present study, we examined the induction of a Ca^{2+}/calmodulin-

dependent isozyme in rat liver and found that the same isozyme is also induced transcriptionally in other organs.

2. MATERIALS AND METHODS

2.1. Materials
L-[2,3-³H]Arginine (spec. act. 55 Ci/mmol, 1 Ci = 37 GBq) was obtained from NEN (DuPont de Nemours, Wilmington, DE). N^G-Monomethyl-L-arginine acetate and (6R)-5,6,7,8-tetrahydro-L-biopterine (H_4BP) were from Calbiochem (La Jolla, CA) and Dr. B. Laboratories (Jona, Switzerland), respectively. Other reagents were of analytical grade and were purchased from Sigma (St. Louis, MO) or Wako (Osaka, Japan).

2.2. Induction of nitric oxide synthase in vivo
Male Sprague-Dawley rats, weighing between 200–300 g, were given heat-killed *Propionibacterium acnes* (25 mg/kg b.w.) through a tail vein, followed five days later by an i.v. injection of *E. coli* LPS (4 mg/kg b.wt.). Organs were isolated from the rats 5 h after LPS injection and frozen at −80°C until use.

2.3. Purification of NO synthase
Frozen organs were homogenized with a Polytron homogenizer in cold buffer A (50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol (DTT), 1 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) using 5 ml of buffer per 1 g of tissue. The homogenate was centrifuged at 105,000 × g for 1 h at 4°C and the cytosol fraction was incubated with 2′,5′-ADP agarose in a ratio of 50-to-1 with gentle agitation for 20 min at 4°C. NO synthase was eluted with buffer A containing 10 mM NADPH. Then 2 ml of pooled material was loaded on an FPLC mono Q HR5/5 column and eluted by a programmed gradient of 0–0.25 M NaCl in buffer A.

2.4. Assay of NO synthase
Samples were added to 100 μl of reaction mixture consisting of 50 mM HEPES, pH 7.5, 20 mM L-[2,3-³H]arginine, 1 mM NADPH, 1 mM EDTA, 1.25 mM calcium acetate, 10 μM FAD, 100 μM tetrahydrobiopterin, 0.1 mM DTT and 1 μg calmodulin. After incubation for 10 min at 25°C, the reaction was terminated by the addition of 1 ml of 20 mM HEPES, pH 5.5, containing 2 mM EDTA and the mixture

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was applied to a Dowex AG50WX-8 (Na⁺ form) column. NO synthase activity was quantified by measuring the radioactivity of the flow-through fraction which contained citrullin but not arginine, essentially based on the method of Bredt and Snyder [3].

2.5. Immunoblotting

Samples were separated by SDS-PAGE in a 6% gel and transferred to Immobilon IPVH (Millipore). The membrane was blocked by over-night incubation with 20% FBS/PBS at 37°C, and was then incubated with 1/250 diluted anti-inducible NO synthase antiserum pretreated with acetone powder of normal rat liver [11]. The immunoblots were incubated with anti-rabbit IgG conjugated with horseradish peroxi-dase, and bands were visualized by incubation in 0.5% H_2O_2 in buffer containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride.

2.6. Northern blot analysis

Total RNA was isolated from each tissue by guanidine thiocyanate extraction, and poly(A)⁺ RNA was separated with Oligotex-dT30 (Japan Roche Co.). Northern blot analysis was performed using a 700 bp fragment of the 5' portion of cloned rat liver inducible NO synthase cDNA (Aduchi et al., to be published elsewhere) as a probe.

3. RESULTS

Administration of killed *Propionibacterium acnes* and LPS to rats results in acute necrosis of the liver [12]. A calmodulin-independent NO synthase activity is reported to be induced during liver necrosis by this treatment [13]. We found that both Ca^{2+}/calmodulin-dependent and -independent NO synthase activities are induced in the liver by this treatment [10]. The former is the only inducible Ca^{2+}/calmodulin-dependent NO synthase so far reported. To determine whether the induction of this Ca^{2+}/calmodulin-dependent isozyme is specific to the liver, we examined the NO synthase activities of various organs after partial purification of the enzyme by 2',5'-ADP-agarose chromatography. As summarized in Table I, this treatment increased the NO synthase activity markedly in the cytoplasms of the lung, spleen and colon, as well as the liver, but not in the cerebrum or cerebellum. The results also showed that a considerable amount of this NO synthase activity is present in the colon without treatment. To character-ize the induced enzyme, we subjected fractions from the liver, lung, spleen and colon obtained by 2',5'-ADP-agarose chromatography to ion exchange column chromatography on a mono Q column. Both Ca^{2+}/calmodulin-dependent and -independent activities were observed in the liver, lung and spleen (Fig. 1) and colon (data not shown). Thus induction of Ca^{2+}/calmodulin-dependent NO synthase is not specific to the liver. From their chromatographic patterns, the Ca^{2+}/calmodulin-dependent isozymes induced in the four organs seemed to be the same.

Previously we prepared polyclonal antibody against the inducible NO synthase from rat liver [11]. In order to characterize the induced NO synthase further, frac-tions after 2',5'-ADP agarose chromatography from these organs with and without treatment with *Propioni-bacterium acnes* and LPS were subjected to SDS-PAGE
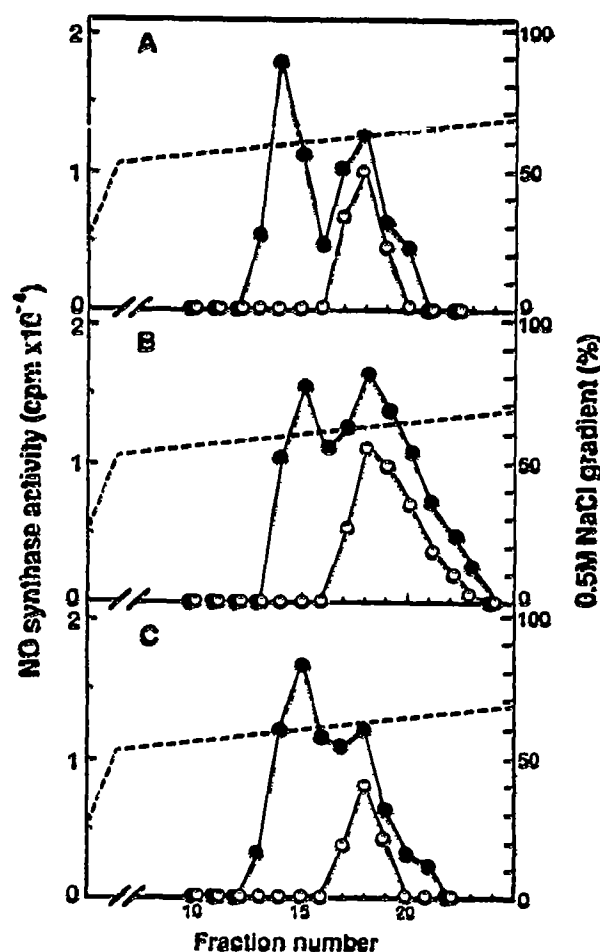

Fig. 1. Chromatographic profiles of NO synthase activity on a mono Q column. Source of NO synthase activity: A, liver; B, lung; C, spleen. NO synthase activity was determined with (●) or without (○) addition of calmodulin to the reaction mixture. The dashed line indicates the concentration of NaCl.
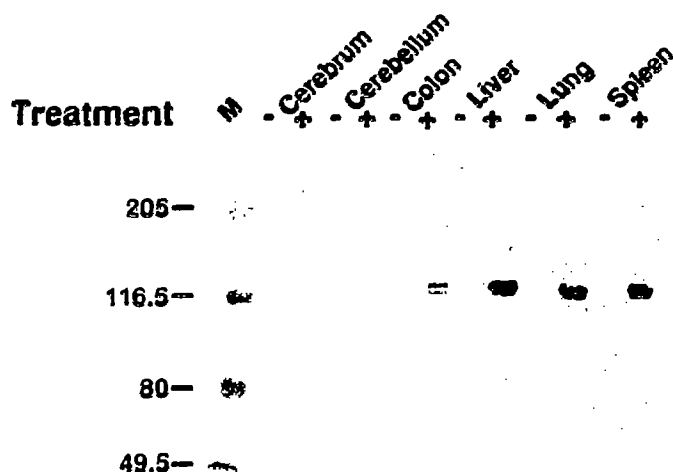

Fig. 2. Western blot analysis of inducible NO synthase in various organs of rats treated with *Propionibacterium acnes* and LPS. Samples of various organs after 2',5'-ADP-agarose chromatography were sub-jected to 6% SDS-PAGE followed by Western blot analysis with anti-body against inducible liver NO synthase [11].

in a 6% gel and examined by Western blotting with this antibody. As shown in Fig. 2, the antibody cross-reacted with the induced NO synthase in the lung, spleen and colon: proteins giving a major band of 125 kDa and a minor band of 120 kDa were observed in these organs only after treatment with *Propionibacterium acnes* and LPS. Amino acid sequencing showed that the protein of 120 kDa was an enzymatically active degradation product of NO synthase that had lost 62 amino acids from the N-terminus of the sequence deduced from the cDNA sequence (to be published elsewhere). The results clearly indicated that the NO synthases induced in the lung, spleen, colon and liver have the same molecular weight. All the above data thus strongly indicate that the same Ca^{2+}/calmodulin-dependent NO synthase is induced in various organs. Western blot analyses of various other organs showed that a similar, probably identical, NO synthase is also induced in the heart, kidney, ileum, colon and adrenal gland, but not in the cerebrum, cerebellum, brain stem, spinal cord or testis (data not shown).

Northern blot analysis was used to examine whether induction of the NO synthase occurred at the transcriptional level. As shown in Fig. 3, before treatment with *Propionibacterium acnes* and LPS, no mRNA was detectable in any of the organs tested, but the treatment resulted in marked induction of mRNA in the lung, spleen, colon, and liver, although not in the cerebrum or cerebellum.

4. DISCUSSION

In the present paper we found that Ca^{2+}/calmodulin-dependent NO synthase can be induced in various organs of rats. There are two types of NO synthase, Ca^{2+}/calmodulin-dependent and -independent types, and all previously isolated Ca^{2+}/calmodulin-dependent isozymes, such as those from brain and endothelial cells, have been considered to be constitutive. The calmodulin dependency of these isozymes might be essential for delicate control of their activity coupled with calcium channels of receptors for specific biologically active substances. On the other hand, inducible-type NO synthases have been considered to be calmodulin-independent. Macrophage NO synthase is a representative of this type. As its biological function is target cell killing [1], delicate control of its activity may not be required. What is the biological function of an inducible Ca^{2+}/calmodulin-dependent isozyme? As showr in the present work, this enzyme seems to be induced in a wide variety of organs upon stimulation with LPS. LPS administration causes endotoxin shock, so maintenance of a sufficient blood flow in major organs after LPS treatment may be difficult. Consequently in this condit on controlled production of nitric oxide to maintain an adequate blood flow and to inhibit intravascular coagulation may be critical for survival. This possibility is
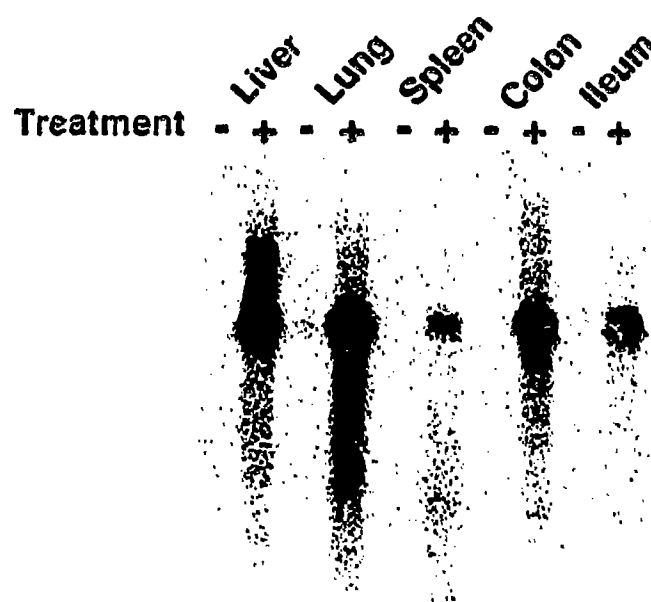
Fig. 3. Northern blot analysis of RNA from rat organs with and without *Propionibacterium acnes* and LPS treatment. Samples of 25 µg of total RNA from various organs were subjected to 1% agarose gel electrophoresis after partial purification on Oligotex dT30.

supported by preliminary immunohistochemical studies on rat organs after LPS treatment, which showed that vascular endothelial cells gave a positive reaction for inducible NO synthase [11].

A Ca^{2+}/calmodulin-dependent NO synthase with a molecular weight of 135 kDa was recently found in a particulate fraction of endothelial cells [14]. The possibility that the inducible Ca^{2+}/calmodulin-dependent enzyme described here is derived from this constitutive enzyme by limited proteolysis is disproved by the finding that, after LPS treatment, the level of the constitutive NO synthase remained constant and its activity remained in the particulate fraction (data not shown). Furthermore, the 135 kDa particulate enzyme differs from our enzyme immunologically (data not shown). Therefore, this inducible Ca^{2+}/calmodulin-dependent enzyme studied in this work is a novel and distinct isozyme [10].

In the present work, we treated rats with both *Propi-*

Table I

NO synthase activities of various organs before and after *P. acnes* and LPS treatment

	Control (cpm/mg protein/min)	*P. acnes*/LPS treated (cpm/mg protein/min)
Liver	120 ± 18	900 ± 180
Lung	210 ± 27	2,360 ± 600
Spleen	148 ± 37	1,860 ± 240
Colon	2,650 ± 15	5,230 ± 230
Cerebrum	13,000 ± 46	8,350 ± 1,530
Cerebellum	14,900 ± 990	12,500 ± 1,400

onibacterium acnes and LPS, but treatment with LPS alone was sufficient to induce both Ca^{2+}/calmodulin-dependent and -independent NO synthase activities (data not shown). LPS treatment is reported to induce calmodulin-independent NO synthase in macrophages [8], but we recently found that at least part of the calmodulin-independent isozyme was a calmodulin-bound form of the Ca^{2+}/calmodulin-depend nt isozyme (Iida et al., to be published elsewhere). It is not clear at present if the calmodulin-independent NO synthase derived from macrophages is different from the calmodulin-independent activity which we studied in the present work.

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