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An inhibitor of inducible nitric oxide synthase ameliorates experimental autoimmune myocarditis in Lewis rats

Taekyun Shin [a,*], Naoyuki Tanuma [b], Seungjoon Kim [a], Jaekwang Jin [a], Changjong Moon [a], Kiok Kim [b], Kuniko Kohyama [b], Yoh Matsumoto [b], Byunghwa Hyun [c]

[a] *Department of Veterinary Medicine, Cheju National University, Cheju 690-756, South Korea*
[b] *Department of Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo 183, Japan*
[c] *Korea Research Institute of Bioscience and Biotechnology, KIST, Taejeon, South Korea*

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Abstract

We studied the effect of nitric oxide (NO) on experimental autoimmune myocarditis (EAC) in rats. We examined the role of inducible nitric oxide synthase (iNOS), an enzyme that produces NO, on hearts affected with EAC, by testing the effects of aminoguanidine (AG), a selective iNOS inhibitor, on the course of EAC. Western blotting detected iNOS in the affected cardiac tissues, but not in CFA immunized cases. Immunohistochemically, the majority of $ED1^+$ macrophages in the EAC lesions were positive for iNOS and nitrotyrosine. A high dose of AG (200 mg/kg/day) significantly reduced the incidence of EAC ($p < 0.05$) and ameliorated the histological score for the cardiac inflammation ($p < 0.01$) compared with the low dose AG (100 mg/kg/day) and vehicle treated groups. The immunoblot analysis showed that a high dose of AG effectively suppressed iNOS in hearts affected with EAC. An iNOS band was barely detected in the high dose AG (200 mg/kg) treated group, while it was distinctively visualized in the vehicle and low dose AG (100 mg/kg) treated groups. These results suggest that iNOS is upregulated in EAC lesions and increased NO production plays an important role in the development of EAC. In addition, selective iNOS inhibitors may have a therapeutic role in treating certain autoimmune diseases including EAC.

Keywords: Experimental autoimmune myocarditis; Nitric oxide synthase; Aminoguanidine

1. Introduction

Nitric oxide (NO) is a readily diffusible nonpolar gas that is formed in a variety of cell types and influences a number of physiological and pathological conditions (Murphy et al., 1993; Nathan and Xie, 1994). The enzyme responsible for NO production exists in two forms: (1) constitutive nitric oxide synthase (cNOS), which is readily activated by agonists that elevate intracellular free Ca^{2+}; and (2) inducible NOS (iNOS), which is induced following several hours of immunological stimulation (Nathan and Xie, 1994). The biological reactions controlled by NO are diverse and include immune cell mediated cytotoxicity, regulation of vascular tone and relaxation, inhibition of platelet aggregation and neural transmission (Moncada et al., 1991). Although NO acts as an intracellular messenger, it can be toxic to cells at high concentrations. Accumulating evidence suggests that NO may be involved in the pathogenesis of autoimmune diseases such as EAE (Lin et al., 1993), rheumatoid arthritis and spontaneous glomerulonephritis (Weinberg et al., 1994).

Experimental autoimmune myocarditis (EAC) is an autoimmune inflammatory cardiac disorder that serves as the animal model for human giant cell myocarditis (Komada et al., 1990, 1992). Although the disease process in EAC is believed to be initiated by autoreactive T cells and macrophages (Komada et al., 1992), the exact mechanism underlying the cardiac injury is not fully understood.

Several reports have provided evidence that NO generated by inflammatory cells including macrophages plays an important role in the pathogenesis of autoimmune disorders, including experimental autoimmune encephalomyelitis (EAE) (MacMicking et al., 1992; Koprowski et al., 1993; Lin et al., 1993; Van Dam et al., 1995; Waldburger et al., 1996) and arthritis (McCartney-Francis et al., 1993).

* Corresponding author. Tel.: +82 64 7543363; fax: +82 64 7563354; e-mail: shint@cheju.cheju.ac.kr

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The selective inhibition of inducible nitric oxide synthase, a key enzyme for NO production, is associated with the amelioration of autoimmune inflammation (McCartney-Francis et al., 1993; Cross et al., 1994; Zhao et al., 1996). The identification of specific mediators, such as nitric oxide in EAC, helps in the study of disease pathogenesis and may suggest possible therapeutic intervention.

This study was designed to investigate whether iNOS, a potent NO production enzyme, is involved in the initiation of EAC and whether an appropriate inhibition of iNOS affects the course of EAC.

2. Materials and methods

2.1. Animals

Lewis rats were obtained from the Korean Research Institute of Bioscience and Biotechnology, KIST (Republic of Korea). They were bred and maintained in our animal facility.

2.2. Purification of cardiac myosin

Cardiac myosin was prepared from the ventricular muscle of human hearts, according to the methods of Murakami et al. (1976). The antigen preparation consisted primarily of a 205 kilodaltons (kd) protein, but contained small amounts of several substances other than cardiac myosin.

2.3. Induction of experimental autoimmune myocarditis

Cardiac myosin fraction was dissolved at a concentration of 2 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl. The antigen solution was mixed an equal volume of complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI) at a concentration of 5 mg/ml (CFA).

Lewis rats 6–8 weeks old were subcutaneously immunized in each hind footpad with 0.1 mg of cardiac myosin in CFA followed by the intraperitoneal injection of 2 μg of pertussis toxin (Sigma). Age- and sex- matched Lewis rats served as controls. They were challenged with PBS in CFA followed by injection with pertussis toxin. The rats were sacrificed under ether anesthesia on days 14, 21, or 28 postimmunization (PI).

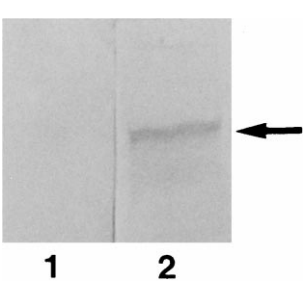

Fig. 1. Immunoblot analysis of cellular proteins with rabbit iNOS antisera. Lane 1 (control) and lane 2 (EAC). In the EAC tissues, the iNOS antisera reacted with a single protein that produced a band corresponding to a molecular weight of approximately 130 kd (lane 2: arrow). No such band was seen in normal heart tissue (lane 1).

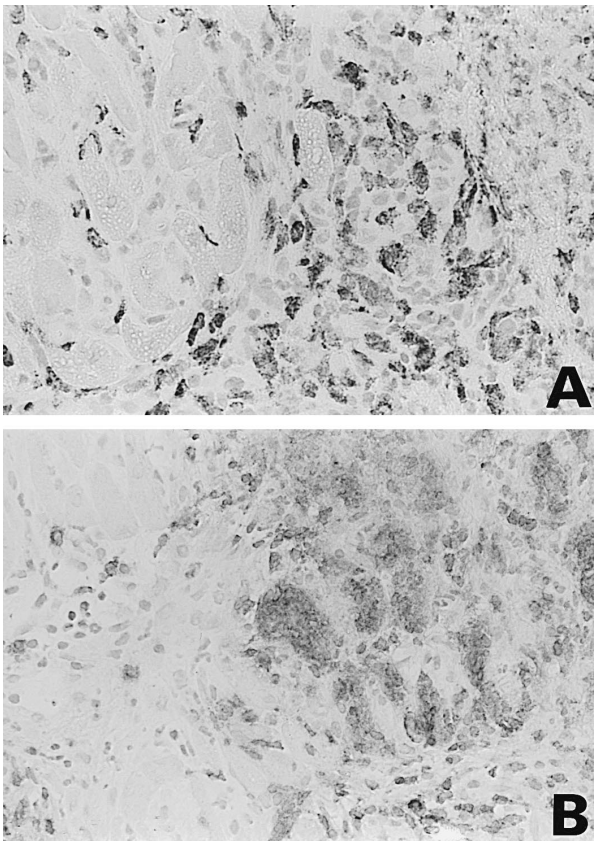

Fig. 2. Immunostaining of ED1 (A) and anti-iNOS antibody (B) in the hearts with EAC at 14 days postimmunization. (A) A large number of inflammatory cells were immunostained with ED1 suggesting that many macrophages are found within EAC lesions. (B) In a serial section immunostained with iNOS ab, most of the macrophages express iNOS. Original magnification: (A) and (B)×132.

2.4. Histopathology

At the time of death, the hearts were examined for the presence of a pericardial effusion and macroscopic findings. The macroscopic findings were graded into three categories: 0, normal; 1, presence of a focal discolored area; 2, presence of a multiple discolored area; 3, presence of a diffuse, multiple discolored area on the cardiac surface. The hearts were removed immediately after death and the heart was fixed in 10% buffered formalin. After the hearts were embedded in paraffin, transverse sections were cut and stained with hematoxylin and eosin. The microscopic finding of myocarditis was scored by three blinded observers as follows: 0 (normal), 1 (a few small lesions), 2

(multiple small lesions or a moderate lesions), 3 (multiple moderate lesions or large lesions) (Komada et al., 1990).

2.5. Antisera and reagents

Antisera employed in this study were rabbit anti-iNOS polyclonal antiserum (Transduction Laboratory, Lexington, CA), rabbit anti-nitrotyrosine polyclonal antiserum (UBI, NY, USA), and ED1 (Serotec, UK) for labeling macrophages. An avidin–biotin complex (ABC) elite kit (Vector, Burlingame, CA) was used for immunohistochemistry. The aminoguanidine was obtained from Sigma. All the materials used for Western blotting were obtained from BioRad (CA, USA).

2.6. Western blot analysis

The rat cardiac tissue was minced, weighed, placed in homogenizing buffer (0.125 M Tris/HCl, pH 6.8, 5% mercaptoethanol, 0.1% Triton X-100, 4 M urea) (1:4 w/v), and homogenized with a tissue homogenizer. The homogenate was sonicated three times (5 s at 4°C), and centrifuged at 14,000 × g for 30 min. The supernatant was diluted with electrophoretic sample buffer, and heated at 95°C for 5 min. Tissue extracts containing 30 μg of total protein were subjected to 7.5% SDS-PAGE according to the methods of Laemmli (1970). The separated proteins were blotted to a nitrocellulose. After blocking nonspecific protein binding by treatment with 5% non-fat dry milk in Tris buffer (pH 7.5) for 1 h, the blots were incubated for 2 h at room temperature in rabbit anti-iNOS antisera (1:1000 dilution in Tris buffer). After three washes in Tris buffer containing 0.1% Triton X-100, the blots were incubated for 1 h with the avidin–biotin complex, and treated with diaminobenzidine according to the manufacturer's instructions (Vector) to develop the color.

2.7. Immunohistochemistry

Immunohistochemical procedures were carried out using an avidin–biotin complex kit (Vector) by following the instruction from the manufacturer. Briefly, 5 μm sections of the heart were deparaffinized, and treated with 0.3% hydrogen peroxide in methyl alcohol for 30 min to block endogenous peroxidase. After three washes in phosphate buffer, the sections were exposed to normal goat serum, and then incubated with each primary antisera optimally diluted in PBS for 1 h at room temperature. After washing, the sections were sequentially treated with biotinylated goat anti-rabbit immunoglobulin and avidin–biotin peroxidase complex, developed with diaminobenzidine–hydrogen peroxidase solution (0.001% 3,3′-diaminbenzidine and 0.01% hydrogen peroxidase in 0.05 M Tris buffer), and finally counterstained with hematoxylin.

2.8. Treatment with AG, an iNOS inhibitor

To evaluate the effect of NO in EAC, rats were treated with AG, an inhibitor of inducible nitric oxide synthase, from day 0 to day 14 PI. The rats were administered a daily dose of either 100 mg/kg or 200 mg/kg of AG dissolved in PBS intraperitoneally. A third group of animals received 200 mg/kg of AG from day 7 to 14 PI. Control rats were treated with PBS only in the same way.

Table 1
Effect of daily intraperitoneal injection of aminoguanidine in rats with experimental autoimmune myocarditis

Parameter	Vehicle		AG (100 mg/kg)	AG (200 mg/kg)	
	(D0–D14 PI)	(D7–D14)	(D0–D14 PI)	(D0–D14 PI)	(D7–D14 PI)
Incidence	9/9	5/5	5/5	2/8*	5/5
Gross grade[a]	2.5 ± 0.3 ($n = 9$)	2.8 ± 0.3 ($n = 5$)	2.5 ± 0.5 ($n = 5$)	0.4 ± 0.4** ($n = 5$)	(3) ($n = 5$)
Pericardial effusion[b]	moderate	moderate	moderate	slight	moderate
histologic score[c]	2.2 ± 0.3 ($n = 6$)	3.0 ± 0.3 ($n = 5$)	3.2 ± 0.2 ($n = 4$)	0.8 ± 0.3** ($n = 5$)	3.3 ± 0.6 ($n = 3$)

The number in parentheses is the number of rats studied.
NE: not evaluated.
*: Significantly different from the other experimental groups including the vehicle-treated control and the low dose AG (100 mg/kg) treated group ($p < 0.05$), revealed by ANOVA with Tukey–Kramer multiple comparisons test.
**: Significantly different from the vehicle-treated control ($p < 0.01$) and the low dose AG (100 mg/kg/day) treated group ($p < 0.01$) during day 0 to 14 postimmunization (PI) as well as the high dose AG (200 mg/kg/day) treated group during days 7–14 PI, revealed by ANOVA with Tukey–Kramer multiple comparisons test.
[a]The score of gross grade was divided into three categories. 0, no gross lesion; 1, presence of a focal discolored area; 2, presence of a multiple discolored area; 3, presence of a diffuse, multiple discolored area on the cardiac surface. The gross grade is expressed as the mean ± S.E.
[b]The pericardial effusion was graded as moderate (> 1.5 ml) and slight (< 1 ml).
[c]The histologic score of myocarditis was scored by three blinded observers as follows: 0 (normal), 1 (a few small lesions), 2 (multiple small lesions or a moderate lesions), 3 (multiple moderate lesions or large lesions). The value was shown as mean ± S.E.

3. Results

3.1. Appearance of iNOS in the course of EAC

Fig. 1 shows the immunoblot analysis for iNOS in EAC at day 14 PI. The iNOS was detected as a single band corresponding to a molecular weight of approximately 130 kd in the EAC tissue (lane 2). No such band was identified in normal heart tissue (lane 1). The iNOS-positive band was consistently found in the inflammatory EAC lesions at days 21 and 28 PI (data not shown).

3.2. Localization of iNOS and nitrotyrosine in EAC

Fig. 2 shows EAC lesions, which are characterized by the infiltration of hematogenous inflammatory cells at days 14 PI. Serial sections immunostained with ED1 and iNOS antisera clearly show many macrophages within the EAC lesions (A) and demonstrate that most macrophages express iNOS (B) and nitrotyrosine, an end product of nitric oxide (results not shown).

3.3. Treatment with an inhibitor of iNOS, AG

The effect of AG on the induction of EAC is summarized in Table 1. A high dose of AG (200 mg/kg/day) significantly reduced the incidence of EAC ($p < 0.05$) (Fig. 3) and ameliorated the histological score for the cardiac inflammation ($p < 0.01$) compared with the low dose AG (100 mg/kg/day) and vehicle treated groups. However, treatment with the same dose of AG (200 mg/kg/day) during the progression stage from days 7 to 14 PI had no effect on the incidence of EAC (Table 1).

To examine whether AG treatment affects the expression of iNOS in the heart, an immunoblot analysis of iNOS was performed with cardiac tissue of cardiac myosin-immunized rats with or without AG treatment. The immunoblot analysis in Fig. 4 shows that a high dose of AG effectively suppresses iNOS in hearts affected with EAC. An iNOS band was barely detected in the high dose AG (200 mg/kg) treated group (Fig. 4, lane 3), while it was distinctively visualized in the vehicle (Fig. 4, lane 1) and low dose AG (100 mg/kg) treated groups (Fig. 4, lane 2).

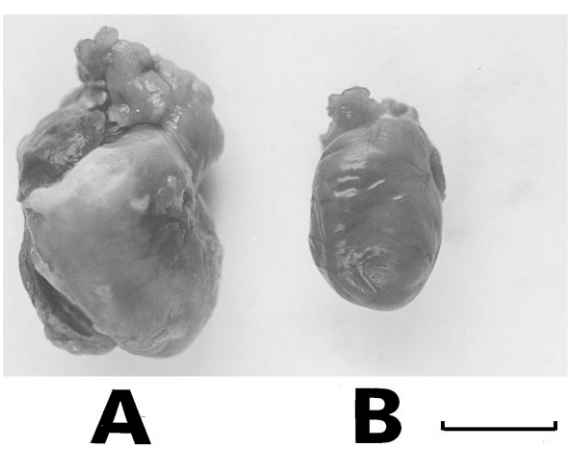

Fig. 3. A representative photo of EAC hearts after vehicle and aminoguanidine treatment. (A) A typical heart with EAC, showing the discolored surface of the heart. (B) The appearance of a cardiac myosin-immunized heart after treatment with AG (200 mg/kg/day) for 14 days. No inflammatory foci are seen on the surface of the heart. (A) and (B) were selected as the most representative cases. Scale represents 1 cm.

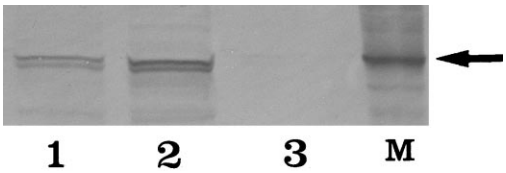

Fig. 4. Immunoblot analysis of iNOS in the affected heart of EAC rats with AG treatment. An iNOS (130 kd) band was barely detected in the high dose AG (200 mg/kg) treated group (lane 3), while it was distinctively visualized in the vehicle (lane 1) and low dose AG (100 mg/kg) treated groups (lane 2). M, iNOS standard marker. Representative data from two different experiments.

4. Discussion

NO acting via iNOS expression has been proposed as one of the important effector molecules in the pathogenesis of disease progression in a variety of animal models of autoimmune disorders, including EAE (MacMicking et al., 1992; Koprowski et al., 1993; Lin et al., 1993; Cross et al., 1994; Van Dam et al., 1995) and rheumatoid arthritis (McCartney-Francis et al., 1993). EAC, an animal model of human giant cell myocarditis, is caused by autoreactive T cells and certain bystander cells including macrophages (Komada et al., 1992). In this study, we confirmed that both iNOS and an end product of nitric oxide, nitrotyrosine, was expressed in the affected heart, suggesting that iNOS plays an important role in damaging the cardiac muscles via increased NO production by inflammatory macrophages.

AG has been used widely to block iNOS activity in disease models involving NO (Cross et al., 1994; Zhao et al., 1996; Brenner et al., 1997). In this study, we found that a high dose of AG (200 mg/kg/day) was necessary to reduce the occurrence of EAC, while a lower dose of AG (100 mg/kg/day) had no effect (Table 1). We did not expect to completely prevent EAC, because EAC is initiated by autoreactive T cells (Komada et al., 1992) and AG does not affect T cell proliferation directly (Brenner et al., 1997).

As far as AG is concerned, we speculate that AG effectively inhibits iNOS in vitro (Misko et al., 1993). Therefore, AG inhibits the generation of NO from iNOS, and ameliorates EAE (Cross et al., 1994; Zhao et al., 1996; Okuda et al., 1997) and EAC (this study). Although AG has been associated with the generation of hydrogen perox-

ide in vitro (Ou and Wolff, 1993), its production in vivo may be minimal and thus does not affect the occurrence of autoimmune diseases including EAE and EAC, which we studied.

There have been several studies addressing the effect of iNOS inhibitors on autoimmune disorders including EAE. Cross et al. (1994) and Zhao et al. (1996) reported that AG effectively ameliorates the progression of EAE in mice and rats, respectively. However, Ruuls et al. (1996) and Gold et al. (1997) reported the contradictory finding that rat EAE was exacerbated by treatment with iNOS inhibitors, although they use different iNOS inhibitors including L-*N*-(1-iminoethyl)lysine (Gold et al., 1997) and *n*-nitro-L-arginine-methylester and *N*-monomethyl-L-arginine (Ruuls et al., 1996).

We postulate that the dose and timing of the administration of the iNOS inhibitor is a critical factor in the treatment of autoimmune disorders. Our findings in the EAC model are largely consistent with those of Okuda et al. (1998), in which AG plays a preventive role in the induction of autoimmune inflammation, and subsequently has detrimental effects on the progression of EAE. In EAE, NO produced by iNOS is known to mediate apoptosis of inflammatory cells leading to recovery (Okuda et al., 1997), as does in vitro T cell apoptosis (Zettl et al., 1997). Furthermore, Gold et al. (1997) reported that endogenously generated NO plays an immunoregulatory role in the T cell response and that the iNOS inhibitor (L-*N*-(1-iminoethyl)lysine) accentuates active EAE.

5. Conclusion

Taking this into consideration, we postulate that the suppression of NO production during the induction stage of EAC prevents EAC by inhibiting the pro-inflammatory role of NO. However, a detrimental effect results from the inhibition of the immuno-suppressive role of NO, after myosin-autoreactive cells have already been generated in the peripheral lymphoid system or after these T cells start to infiltrate the pericardial sac. Thus, both the dose and timing of selective iNOS inhibition are important in the treatment of autoimmune diseases such as EAC and EAE.

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